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14. ABSTRACT

The Wnt/ß-catenin pathway has been extensively studied for its role in development and cancer. The canonical Wnt signal is transduced by ß-catenin, which acts as a transcriptional coactivator by associating with the Tcf/LEF family of transcription factors. It has been established that Wnt signaling regulates the self-renewal of normal stem cells in both the hematopoietic systems and the epidermis. In addition, constitutive activation of the Wnt pathway has been implicated in a number of epithelial cancers, possibly by promoting stem cell survival. However, the importance of this pathway in breast stem/progenitor cells has not yet been elucidated.
Objective/Hypothesis: The central hypothesis of this study is that the Wnt/ß-catenin pathway plays a critical role in mammary gland stem cell survival, and as a result promotes tumorigenesis and resistance to conventional therapies.

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Table of Contents

Introduction

Breast epithelium is capable of completely and functionally regenerating upon transplantation. This impressive renewal capacity has been ascribed to the function of a multipotent mammary gland stem/progenitor cell population that resides and persists throughout the mammary parenchyma. Mutations or epigenetic changes either in long-term stem cells or their immediate progeny, the transit amplifying multipotent progenitors, have been suggested to be the foundation of malignancy (*1*). Elucidating the role of stem/progenitor cells in preneoplasia may be crucial to understanding the etiology of breast cancer, and may lead to better chemopreventative strategies.

Body

Specific Aim I: *To study the role of ß-catenin in the regulation of breast stem/progenitor cell survival and differentiation in vivo.*

1) Accomplished – *To generate reconstituted mammary gland from primary mammary epithelial cells (MECs) expressing stabilized ß-catenin*

Transducing primary mammary epithelial cells (MECs) using Ad-Cre1 has been previously established in our laboratory using MECs derived from the R26R reporter mice that express ß-galactosidase upon Cre-mediated recombination (*2*). Tissue was dissociated with collagenase and trypsin and enriched for epithelial cells as described by Pullan and Streuli (*3*). To mark the cells that have been successfully recombined, mice carrying the R26R reporter construct were crossed to the $Catnb^{lox(ex3)}$ mice, and virgin mammary gland tissue from all 10 glands were taken. MECs derived from both the R26R mice as well the compound $Catnb^{lox(ex3)}$/R26R mice were analyzed for Cre-mediated recombination by X-gal staining 2 days after viral transduction. Similar to the previously published study (*2*) at MOI of 40, 75-80% of the Ad-Cre1 transduced cells from the R26R reporter MECs stained positively for ß-galactosidase (Figure 1a, FR). Comparable transduction efficiency was found in the MECs derived from the compound $Catnb^{lox(ex3)}$/R26R mice (~75-80%) (Figure 1a, DF). These results indicate that recombination has occurred in the majority of the primary MECs.

To confirm that Ad-Cre1 mediated recombination occurred at the Catnb locus as well as the R26R locus, we performed PCR analysis as well as RT-PCR to determine whether the β-catenin exon 3 was deleted at both the DNA and the RNA level. PCR analysis of the loxP sites flanking the β-catenin exon 3 fragment from primary MECs indicated that recombination has occurred. Both the wild-type and the recombined fragments were detected by PCR (Figure 1b). RT-PCR analysis confirmed that the primary MECs expressed the mutant ß-catenin mRNA (Figure 1c). In addition, to determine that the mutant ß-catenin is functional, we examined the expression of a number of known ß-catenin targets, such as cyclin D1 and ITF2 as read-outs of the ß-catenin signaling (*4-8*). By RT-PCR, we have shown that cyclin D1 and ITF2 are upregulated in the transduced $Catnb^{lox(ex3)}$ primary MECs (Figure1d and e).

Having established that stabilized ß-catenin is expressed following Ad-Cre1 mediated recombination in a majority of the primary mammary epithelial cells, presumably in a mixture of both differentiated and progenitor cells, we then determined whether stabilized ß-catenin affects mammary gland outgrowth. Following transduction with either Ad-ß-gal as a control or Ad-Cre1, approximately $1X10^6$ $Catnb^{lox(ex3)}$ MECs were transplanted in to the cleared inguinal (#4) fat pads of 3-week-old athymic nude

female mice. Transplants were analyzed between 8-12 weeks post transplantation for the presence of outgrowths (Figure 2a). Seven out of 7 of the control MECs (transduced with Ad-ß-gal) reconstituted mammary glands. However, 0 out of 7 of floxed ß-catenin transplants (transduced with Ad-Cre1) were able to grow out. PCR on DNA from the reconstituted mammary glands showed that recombination in exon 3 has occurred in the Ad-Cre1 treated (floxed) ß-catenin transplants (Figure 2b). These data suggest that stabilizing ß-catenin using the Ad-Cre1 affects the ability of mammary gland stem cells to give rise to mammary gland outgrowths. ß-catenin may be important in mammary progenitor cell survival, and may have an inhibitory effect when it is stabilized. The toxicity from the Adenovirus is minimal. Primary wild type MECs treated with Ad-Cre1 grew out as efficiently as wild type MECs without Ad-Cre1 (data not shown), suggesting that the toxicity from the Adenovirus is minimal. Therefore, the lack of outgrowths from the floxed cells is most likely not due to Ad-Cre1.

To determine at what stage the expression of stabilized ß-catenin is potentially blocking outgrowths, we examined transplants at early time points, 2, 3, and 5 weeks after transplantation surgery using primary MECs derived from 8-10 week old $Catnb^{lox(ex3)}$ female mice. Following transduction with control, Ad-ß-gal, or Ad-Cre1, approximately $1X10^6$ cells were injected into the cleared inguinal fat pad of 3-week-old female athymic nude mice. The outgrowths were analyzed at 2, 3, and 5 weeks after transplantation surgery. Three out of 3 of the control cells grew out, at 2, 3, and 5 weeks (Figure 3b, d, and f). The extent of outgrowths increased gradually from 2 weeks to 5 weeks as expected. In contrast, the floxed ß-catenin cells had limited outgrowths at 2 and 3 weeks compared to their control counterparts (Figure 3a and c), and at 5 weeks (Figure 3f), we did not observe any outgrowths. These results suggest that expression of ß-catenin is delicately regulated at early stages of mammary gland development and that in contrast to overexpression by hormone dependent promoters such as WAP or MMTV, stabilizing the expression of endogenous ß-catenin inhibits virgin ductal development.

Dysregulation of the Wnt/ß-catenin pathway has been associated with tumorigenesis in a number of tissues, including the prostate, the intestine, as well as in the breast (*9, 10*). Recent studies by Liu et al have demonstrated that Wnt effectors induce the accumulation of mammary epithelial progenitors defined by their SP phenotype as a surrogate stem cell marker, both in vivo and in vitro (*11*). In addition, recent reports demonstrated that Wnt-activating ß-catenin mutation supported oncogenesis in cooperation with simultaneous dysregulation of another oncogenic pathway, such as the Ras (*12*) or Notch (*13*) pathways, resulting in carcinogenesis. Moreover, the upregulation of ß-catenin signaling accounted for clonal expansion of hyperplastic cells, which may be an early event in tumorigenesis. Besides oncogenesis, misregulation of ß-catenin may induce cells to transdifferentiate into other cell types. Boerboom et al showed that overexpression of ß-catenin in the mouse overy, specifically the granulose cells, causes late onset granulose cell tumor (*14*). Within the pretumoral lesions, the researchers observed an upregulation of the inhibitors of the Wnt pathway, such as DKK, Wip1, and Axin2, as well as upregulation of both osseus and neuronal markers, suggesting that overexpression of ß-catenin in the granulose cells leads to transdifferentiation of the granulose cells into other cell types. Therefore, by expressing stabilized ß-catenin in the mammary epithelial cells, upregulation of Wnt inhibitors may inhibit normal mammary outgrowth while altering the cell fate of these transplanted

epithelial cells. Further studies to examine the expression profiles of the MECs stabilized ß-catenin will determine whether Wnt inhibitors are upregulated in these cells, and whether markers of other cell types are upregulated.

In contrast to ß-catenin's role in oncogenesis, during development, ß-catenin may be important in regulating the stem cell niche. Studies in C. Elegans has shown that ß-catenin is required in transducing the Wnt/MAPK signal in the specification of distal tip cells which in hermaphrodites and males are considered the stem cell niche (*15*). ß-catenin may also be an important component in the hematopoietic stem cell (HSC) niche. These authors show that the asymmetrical localization of N-cadherin and ß-catenin between a subpopulation of osteoblasts and the long-term HSCs determine the number of long-term HSCs within the niche (*16*). Finally, recent studies have shown that a subtle balance with respect to the temporal regulation, location, as well as the level of ß-catenin is critical in specifying cell fate within the stem cell niche of the hair follicles (*17*). While high levels of ß-catenin expression affects hair follicle formation, and repression of the ß-catenin signal results in differentiation into sebocytes and interfollicular epidermis (*18*) sustained low levels of ß-catenin results in mobilization of the stem cells of the bulge, followed by precocious entry into a new phase of hair follicle growth (*19*). In contrast, ablation of ß-catenin in the skin results in a loss of bulge stem cells in the stem cell niche, an expansion in transit amplifying cells that will transdifferentiate into sebocytes and interfollicular epidermis independent of stem cells (*19*). Therefore, the level of expression, the location, as well as the temporal regulation of ß-catenin, may have differential effect within the stem cell niche and impact lineage determination and differentiation differently. In attempting to interpret the transplant results we have come to the conclusion that stabilizing endogenous ß-catenin by Cre-recombination produces an imbalance of ß-catenin within the stem cell niche, and therefore, may inhibit differentiation of those stem cells into mammary epithelial cells.

2) Accomplished – *To analyze effects of stem cell renewal by subsequent serial tissue transplantation from the initial reconstituted mammary gland.*

We initially proposed to analyze the effects of stem cell renewal by in vivo serial tissue transplants derived from the primary mammary gland reconstitution experiment. However, since the floxed primary MECs failed to reconstitute a cleared fat pad, the original proposal of serial tissue transplants in vivo would not work. Alternatively, we have opted to use an in vitro mammosphere assay to study the effect of multipotent stem/progenitor cell self-renewal.

We utilized the mammosphere in vitro self-renewal assay described by Dontu et al (*20*), which is based upon the hypothesis that progenitor cells are able to survive in anchorage-independent conditions. By retroviral tagging experiments, Dontu et al. have previously shown that mammospheres are clonally derived, and not a result of aggregation (*20*). In addition, mammospheres are now known to comprise a heterogeneous population of cells, with multipotent mammary stem/progenitor cells within the core, and surrounded by progenitor cells in various stages of differentiation.

Furthermore, we have chosen the COMMA-D ß-geo (CDßgeo) murine mammary cell line to examine the self-renewal efficiency of the progenitor population using stem cell antigen 1 (Sca1) to enrich for multipotent progenitor cells. The CDßgeo cell line is a clonal, immortalized cell line isolated from the parent COMMA-D (CD), preneoplastic,

mouse mammary cell line (*21*). The CD cell line has been shown to contain mutations in both alleles of p53 (*22*). Recent studies by Deugnier et al have demonstrated that CDßgeo cells contain a population of multipotent progenitor cells that express putative stem cell markers, CD49f, CD24, and Sca1. They have shown that $Sca1^+$ or high cells are enriched in outgrowth potential. The outgrowths from $Sca1^+$ cells form terminal end buds, ducts, and alveolar structures in pregnant hosts (*21*). In a separate manuscript where we examined the primary mouse mammary progenitor cells (Appendix 1) the data from the primary mouse cells are consistent with the observation made using the CDßgeo cells. Thus, it is an extremely tractable cell model, and the results presented are entirely consistent with the results from primary cell.

We examined the expression patterns of putative stem cell markers in the secondary mammospheres, such as CD49f, TIE2, Keratin 6 (K6), and epithelial specific markers Keratin 14 (K14). In previous studies, CD49f has been associated with multi-potent mammary gland progenitors (*23*), TIE2 has been shown to identify quiescent hematopoietic stem cells and is thought to function by maintaining these cells in the bone marrow niche (*24*). K6 is expressed within the body cells of the developing mammary terminal end buds, and overexpressed in Wnt-1 murine mammary tumors together with Sca1 (*25, 26*). K14 is an epithelial-specific marker used to identify basal epithelial cells while K18 identifies luminal epithelial cells.

Ten thousand cells were plated on low adherence plates in serum free media supplemented with EGF and bFGF as growth stimulants. Approximately 0.1-0.6% of the CDßgeo cells were able to form mammospheres. In the secondary mammospheres examined, CD49f was expressed mainly in the center of the mammosphere (Figure 4b). K14 staining was also localized in the center of the mammosphere (Figure 4c). Both TIE2 and K6 were randomly distributed throughout the mammospheres (Figure 4d, e).

3) Accomplished – *To analyze morphology by whole-mount and histological analysis.*

Mammary gland morphology was analyzed in detail in the control and the floxed transplants 8 weeks after transplantation. The control whole mounts showed outgrowth (Figure 2a, –Ad-Cre1). Detailed description of the floxed transplants have been reported in Specific Aim I, page 5. Histological analysis of the control transplants indicates that the control cells organized into two layers containing luminal and myoepithelial cells (Figure 2d).

4) Accomplished – *To study the effect of stabilizing ß-catenin on cell differentiation using the mammary gland reconstitution system.*

We hypothesized that using a mammary specific cell model, such as the COMMA-D ßgeo (CDßgeo) cells, where we can overexpress proteins of interest, expressing stabilized ß-catenin might enhance mammary outgrowths, while inhibition of ß-catenin signaling through expression of the dominant-negative construct ß-engrailed might inhibit or delay outgrowth. To examine the effect of the ßcatenin signaling on the ability to form outgrowths we transduced CD cells with the ßgalactosidase (control), stabilized ß-catenin, and ß-engrailed constructs cloned into pS2 vectors previously described (*27*) and transplanted these cells into the cleared fat pads of 3 week-old Balb/c

mice. Eight fat pads (4 mice) were transplanted per construct. An initial biopsy (n = 2 per construct) was taken for whole mount staining at 8 weeks.

Outgrowths from mice transplanted with ß-catenin-transduced cells were consistently more prominent and filled a larger percentage of the fat pad (Figure 5 middle panel) (eight-week outgrowths). Although a small number of tumors developed from cells transduced with each of the three constructs by twenty weeks, ß-catenin-derived outgrowths which produced tumors developed consistently invasive, high-grade tumors, and all ß-catenin derived outgrowths after 8 weeks demonstrated evidence of mammary intraepithelial neoplasia (MIN, Figure 5 - ß-catenin H&E inset).

5) Accomplished – *To analyze regulation of downstream target genes by immunohistochemical analysis.*

Immunohistochemical analysis using Ki67, a proliferation marker, indicated that the control cells were actively cycling (Figure 2i) This was confirmed by using cyclin D1. The floxed ß-catenin transplants either did not grow out, or had very limited outgrowth lacking any obvious mammary gland structure (Figure 2a, Stabilized ß-catenin). Analysis of H&E staining of sections from the floxed ß-catenin transplants demonstrated that primary MECs expressing stabilized ß-catenin were able to form two distinct cell types: luminal and myoepithelial cells (Figure 2d). Using the proliferation markers Ki67 and cyclin D1, we observed that the cells from the floxed ß-catenin transplants are not proliferative when compared to the control transplants (Figure 2g and h). These results suggest that stabilized ß-catenin may have an inhibitory role on proliferation, and that may be one of the reasons the floxed ß-catenin transplants are unable to repopulate a cleared fat pad upon transplantation.

Specific aim II: To analyze lineage markers in cells expressing stabilized ß-catenin

1) Accomplished – To use FACS analysis to profile changes in the stem/progenitor cell populations in primary MECs that express stabilized ß-catenin

We chose to use the side population (SP) cells, which efflux Hoechst dye, as a surrogate marker of stem cells (*28*). Consistently, cells expressing the stabilized mutant ßcatenin by using the loxP/Adeno-Cre contained more SP cells than controls up to two fold. Further characterization of SP cells with respect to radiation resistance is discussed in Specific Aim III, page 10.

2) Accomplished – *To analyze the effect of ß-catenin on stem cell survival by sorting for cell surface markers implicated in tumorigenesis and metastasis*

We have observed an expansion of SP when primary MECs are grown in culture. This may be due to the activation of the multi-drug pumps such as ABCG2 that are upregulated in the presence of growth factors in the culture media. However, the stem/progenitor potential within the cultured primary MECs do not appear to be compromised by culturing, since we are able to use the cultured primary MECs for our transplant experiments.

CD44+/CD24– has been used by Wicha et al as cancer stem cell markers (*29*). We have observed that both CD44 and CD24 are present in the wild type primary MECs (Figure 6a), although data in our lab indicates that CD24 appear to be present at higher

levels in tumor cell lines (not shown). How CD44+/CD24– in the wild type MECs behave compared to the cancer stem cell markers is not known. Experiments are on-going to determine the clonogenic and perhaps tumorigenic potential of these CD44+/CD24– cells. The other two potential stem cell markers that we have examined are Sca1 and cKIT. Both have been used extensively in the hematopoietics field to identify bone marrow stem cells. We have observed that primary MECs express Sca1, but not cKIT (Figure 6b). The reason we are not able to find cKIT positive cells may be due to the inherent cell preparation procedure that chews up cKIT's labile surface marker. The advantage of employing Sca1 as an alternative stem cell marker is that we can use it to isolate stem/progenitor cells without the toxicity of the Hoechst dye. Further characterization of Sca1 subpopulation with respect to radiation resistance is described in Specific Aim III, page 10.

3) Accomplished – *Attempt to enrich for stem cells by growing primary mammary cultures under non-adherent conditions, and to study the effect of stabilized ß-catenin in stem cell enrichment by FACS sorting for stem cell markers.*

Studies using transgenic mice expressing Wnt1 or stabilized ß-catenin, such as the MMTV-Wnt1 and MMTV-DN89b-catenin, have indicated that hyperplasias and tumors rapidly develop and that they are enriched in cells expressing stem and/or progenitor markers (*25, 30*). Additional studies from our laboratory using a dominant negative chimera, ß-engrailed, which specifically suppressed ß-catenin signaling without affecting its cell-cell adhesion function, inhibited cell survival in lobuloalveolar progenitors (*27*). These studies suggested that ß-catenin plays a critical role in stem or multipotent progenitor cell self-renewal.

To determine if there is any difference in self-renewing efficiency between $Sca1^+$ and $Sca1^-$ subpopulations, and to examine whether ß-catenin is required for self-renewal, we first transduced the CDßgeo cells with control (MSCV-IRES-GFP), stabilized ß-catenin (MSCV-ß-catenin-IRES-GFP), or the dominant-negative chimera, ß-engrailed (MSCV-ß-engrailed-IRES-GFP), and then sorted into $Sca1^+$ and $Sca1^-$ populations. The efficiency of secondary mammosphere formation (the number of mammospheres per the number of seeded cells) generated by both the $Sca1^+$ and $Sca1^-$ populations was quantitated as described in Materials and Methods (Appendix 2). The control $Sca1^+$ population demonstrated a significant increase in the efficiency for mammosphere formation compared to the control $Sca1^-$ population (GFP control $Sca1^+$ vs. $Sca1^-$ 1.9 - fold, $*p < 0.02$, Figure 4f). In addition, transduction with stabilized ß-catenin enhanced $Sca1^+$ mammosphere formation compared to the GFP control (GFP $Sca1^+$ vs. ß-catenin $Sca1^+$, 1.6- fold increase, $**p < 0.008$, Figure 4f), while ß-engrailed decreased the number of mammospheres in both the $Sca1^+$ and the $Sca1^-$ populations (5.4-fold decrease, $p < 0.004$, Figure 4f). These data indicate that stabilized ß-catenin selectively enhances the mammosphere-forming capacity in the $Sca1^+$ cells while the dominant-negative ß-engrailed depletes the mammosphere-forming capacity.

Specific Aim III: To determine if stabilized ß-catenin protects stem cells against druginduced apoptosis

1) Accomplished – *to determine if stabilized ß-catenin protects stem cells against radiation induced cell death*

To test the hypothesis that stem-like progenitor cells in the mammary gland are resistant to radiation compared to the non-stem cells, we used the Hoechst dye effluxing Side Population (SP) and Stem Cell Antigen-1 (Sca1) as two surrogate stem cell markers that separate these two populations of cells. As previously reported, we have observed an increase in %SP following radiation in Balb/c primary mammary epithelial cells (Figure 7a). To determine whether the expansion observed in the SP is consistent in expansion in stem/progenitor populations, we used Sca1, and observed a similar expansion of $Sca1^+$ cells following radiation. Interestingly, the number of $Sca1^-$ cells decreased following radiation (Figure 7b).

Phosphorylation at double stranded breaks following radiation is one of the earliest responses to radiation-induced DNA damage. Using a fluorescent antibody specific for the phospho-H2AX, discrete nuclear foci can be visualized and quantitated following radiation (*31*). To determine whether mammary epithelial stem cells may be affected by DNA damage, we used phospho-H2AX to compare DNA damage between the $Sca1^+$ and $Sca1^-$ cells. By quantitating the number of phospho-H2AX foci in the $Sca1^+$ and the $Sca1^-$ cells, we observed that following radiation at 2 Gy, the $Sca1^-$ cells displayed 3.5 fold or more foci compared to the $Sca1^+$ cells (Figure 8e). In addition, by quantitating the number of cells containing foci, we observed that following radiation the number of $Sca1^-$ cells increased 2 fold while the number of $Sca1^+$ cells remained the same after radiation (Figure 8f) Taken together, this demonstrated that the $Sca1^+$ cells show less DNA-damage foci following radiation than $Sca1^-$ cells, suggesting that this may be one mechanism the $Sca1^+$ cells evade radiation-induced damage.

It is well known that rather than apoptosis or necrosis, radiation induces mitotic cell catastrophe. To determine whether radiation affects $Sca1^+$ cells and $Sca1^-$ cells differently, we examined the cell cycle profiles of $Sca1^+$ and $Sca1^-$ cells, focusing on the G0 and G1 phases. Before radiation, we observed that $Sca1^+$ cells were mostly in G1 and S/G2/M phases of the cell cycle, whereas the $Sca1^-$ cells were in G0. Following radiation, we observed that the $Sca1^+$ cells were pushed towards S/G2/M phases, while the $Sca1^-$ cells gradually increased in G1 (Figure 9). This demonstrated that the $Sca1^+$ cells were in distinctly different phases of the cell cycle than $Sca1^-$ cells. In addition, following radiation, the $Sca1^+$ cells were able to cycle, and seemed to be unaffected by radiation. The $Sca1^-$ cells increased in G1, but decreased in S/G2/M, suggesting that this population may be undergoing radiation induced mitotic catastrophe.

Next, we determined the clonogenicity of $Sca1^+$ and $Sca1^-$ cells. Clonogenic cells are defined as those neoplastic cells within a tumor that have the capacity to produce an expanding colony of descendants, and therefore, the capacity to regrow the tumor if left intact at the end of treatment (*32*). We sorted cells into $Sca1^+$ and $Sca1^-$ populations directly into 96well plates containing growth factor reduced Matrigel in equal numbers, and their clonogencity were assessed after 10 days. The $Sca1^+$ cells were able to form 13.8±1.6 clonogens, and the numbers were not affected following radiation, 16±3 clonogens. The $Sca1^-$ cells formed fewer clonogens, 4.6±1, than the $Sca1^+$ cells, and the numbers of clonogens decreased following radiation 3.2±0.7 (Figure 10). This data demonstrates that the $Sca1^+$ cells are more clonogenic than $Sca1^-$ cells and clonogen formation is resistant to radiation.

2) Accomplished – *To determine the mechanisms by which ß-catenin acts as a survival factor to enhance stem cell survival.*

We examined the role of Wnt/ ß-catenin signaling in response to radiation in wild-type MECs from Balb/c mice with flow cytometry on fixed cells. Staining with the anti-non-phospho- ß-catenin-PE antibody which binds to activated ß-catenin showed that ß-catenin is selectively activated in $Sca1^+$ cells in response to radiation, whereas ß-cat staining in $Sca1^-$ cells is unchanged in response to radiation (Figure 11a). Survivin, a bifunctional member of the inhibitor of apoptosis gene family, has been shown to be upregulated by TCF/ß-catenin in intestinal progenitor cells upon UV-B irradiation (*33*). In addition, survivin has been shown to play an essential role in mitosis, in both the segregation of sister chromatids and the assembly stabilization of microtubules in late mitosis (*34*). This suggests that overexpression of ß-catenin may enhance cell survival on radiation treatment at least in part by regulating survivin. Using real-time PCR, we demonstrated that survivin mRNA expression was selectively enhanced in $Sca1^+$ cells in response to radiation ($p = 0.01$; Figure 11b). Taken together, this demonstrated that the Wnt/ß-catenin pathway is involved in increased survival of stem/progenitor cells following radiation treatment.

In a separate manuscript we have examined the radioresistance of CDßgeo cells (Appendix 2), and the data from the CDßgeo cells are consistent with the observations made in the primary mouse cells. Thus, this is an extremely useful tractable cell model that can be used for high throughput screening to manipulate signaling pathways that regulate progenitor cell fate.

We also observed consistent results using the CDßgeo cells. To further characterize the intrinsic difference between the $Sca1^+$ and $Sca1^-$ cells, we compared the level of ß-catenin in these two subpopulations. Using an antibody specific for the non-phosphorylated (or active) form of ß-catenin, we compared the level of ß-catenin using flow cytometry analysis (Figure 12 a). There is a significantly lower percentage of cells with detectable levels of non-phosphorylated ß-catenin in the $Sca1^-$ cells (~1.2%) as compared to the $Sca1^+$ cells (~95%) (Figure 12a). Deconvolution microscopy revealed that non-phosphorylated ß-catenin is mainly localized around the cell membrane in the $Sca1^+$ cells, whereas it is difficult to detect ß-catenin in the $Sca1^-$ cells. Following radiation at 4 Gy, the non-phosphorylated ß-catenin is found mainly in and around the nucleus rather than around the cell membrane. It appears that $Sca1^+$ and $Sca1^-$ cells are intrinsically different with respect to the level of non-phosphorylated ß-catenin. Interestingly, the localization of ß-catenin was altered dramatically following radiation in the $Sca1^+$ cells (Figure 12b)

3) Accomplished – *To study the sensitivity of primary mouse mammary epithelial cells to radiation in vivo*

Welm et al (*35*) showed that the SP population is enriched for $Sca1^+$ cells and that $Sca1^+$ progenitors give rise to outgrowths when transplanted. In our study, the percentage of $Sca1^+$ cells within the SP increases with radiation while the percentage of $Sca1^-$ cells is selectively decreased with radiation (Figure 7b) Stingl et al recently reported very high levels of $Sca1^+$ cells after culture of primary MECs similar to the methods used in this study and as opposed to analysis of freshly isolated MECs (*23*). To rule out the possibility that the increase in $Sca1^+$ cells was a result of performing

radiation treatment of primary cultures of these cells, we radiated the mammary glands of anesthetized Balb/c mice *in vivo*, dissected the glands and performed Sca1 analysis on freshly dissociated MECs (Figure 13a) *In vivo* radiation (4 Gy) significantly decreased the percentage of $Sca1^-$ cells (88% 0 Gy vs 70% 4 Gy $p < 0.0001$) and increased the percentage of $Sca1^+$ cells (12% vs 30%, $p < 0.0001$). The percentage of $Sca1^+$ cells after fresh digestion ranged from 10-25% and consistently increased approximately 3-fold after 4 Gy (n =3).

The $CD24^+CD29^+$ population recently characterized for its ability to give rise to mammary outgrowths from a single cell (*36*) was examined in parallel using freshly isolated MECs. Using freshly isolated MECs, we observed a level of $CD24^+CD29^+$ cells similar to that published by Shackleton et al (< 10%). *In vivo* radiation (4 Gy) did not enrich for this stem cell population and in fact decreased this population by approximately one-third ($lin^-CD24^+CD29^+$ 12.5% 0 Gy vs. 8% 4Gy, $p = 0.01$, Figure 13b). Radiation decreased the brightest double positive cells in this population by approximately two-thirds ($p = 0.002$, Figure 13b). For comparison, the radiation resistance of this population was also examined in MCF-7 cells. Radiation dramatically increased the $lin^-CD24^+CD29^+$ population in MCF-7 cells (34% 0 Gy vs. 53% 2 Gy and 71% 4 Gy, $p = 0.003$ 0 Gy vs. 2Gy and $p = 0.0002$ 0 Gy vs. 4 Gy, Figure 13c). Both the $CD24^+CD29^{lo}$ and double negative populations were significantly diminished after irradiation.

Key Research Accomplishments

- **Mercy S. Chen***, Wendy A. Woodward*, Fariba Behbod, Jeffrey M. Rosen. On mammary stem cells. J. Cell Science 2005 Aug 15;118(Pt 16):3585-94.
  *These authors contributed equally

- **Mercy S. Chen***, Wendy A. Woodward*, Fariba Behbod, Maria P. Alfaro, Thomas Buchholz, and Jeffrey M. Rosen. Wnt/ß-catenin-Mediated Radiation Resistance of Mouse Mammary Stem-like/Progenitor Cells (Submitted).
  *These authors contributed equally

- **Mercy S. Chen***, Wendy A. Woodward*, Fariba Behbod, Maria P. Alfaro, and Jeffrey M. Rosen. Wnt/ß-Catenin Mediates Radiation Resistance of Stem Cell Antigen-1 Positive Progenitors in an Immortalized Mammary Gland Cell Line (Submitted).

Reportable Outcomes

Mercy S. Chen was awarded the degree of Doctor of Philosophy in Cell Biology on March 31, 2006.

Meeting abstracts:

Mercy S. Chen, Wendy A. Woodward, Fariba Behbod, Maria P. Alfaro, Thomas Buchholz, and Jeffrey M. Rosen. Wnt/ß-catenin-Mediated Radiation Resistance of Mouse Mammary Stem-like/Progenitor Cells. Keystone Symposium Stem Cells, Senescence and Cancer. Singapore, 2005.

Mercy S. Chen, Wendy A. Woodward, Fariba Behbod, Maria P. Alfaro, Thomas Buchholz, and Jeffrey M. Rosen. Wnt/ß-catenin-Mediated Radiation Resistance of Mouse Mammary Stem-like/Progenitor Cells. Baylor Breast Center Retreat, 2005.

Mercy S. Chen, Wendy A. Woodward, Jeffrey M. Rosen. ß-catenin: A Pivotal Role in Mammary Gland Stem Cell Survival and Differentiation. DOD Era of Hope meeting. Philadelphia, 2005. Oral Presentation.

Mercy S. Chen, Wendy A. Woodward, Jeffrey M. Rosen. ß-Catenin: A Pivotal Role in Mammary Gland Stem Cell Survival and Differentiation. Gordon Research Conference on Mammary Gland Biology. Italy, 2004.

Conclusion

Stem/progenitor cells possess many of the features that constitute the tumor phenotype, including self-renewal and essentially unlimited replicative potential (*37*). The mutations that initiate breast cancer appear to accumulate slowly in cells that persist throughout the patient's lifetime, as suggested by the exponential increase of breast cancer incidence with age (*38*). Stem/progenitor cells are thought to be targets of tumorigenesis and may acquire self-renewal properties due to activation of signaling pathways, such as the Wnt/ß-catenin pathway, known to be critical for stem cell self-renewal and maintenance.

The Wnt/ß-catenin pathway has been extensively studied for its role in development and cancer. The canonical Wnt signal is transduced by ß-catenin, which acts as a transcriptional coactivator by associating with the Tcf/LEF family of transcription factors. It has been established that Wnt signaling regulates the self-renewal of normal stem cells in both the hematopoietic systems and the epidermis (*28, 39*). In addition, constitutive activation of the Wnt pathway has been implicated in a number of epithelial cancers, possibly by promoting stem cell survival. Properties such as self-renewal and lineage specification as well as the signaling pathways important in controlling these properties appear to be shared between the normal and cancer stem cells (*37*). Therefore, we hypothesized that progenitor cells may be resistant to conventional cancer therapy, such as radiation, and thus, may be responsible for recurrent disease. An understanding of the Wnt/ß-catenin pathway in regulating mammary gland stem cells may provide new insights into the etiology of breast cancer, as well as the rational design of improved treatment for breast cancer.

Based on the studies presented here, we believe that the ß-catenin signal transduction pathway is critical for breast stem cell survival. This signaling pathway has also been shown to be critical in cancer stem cell maintenance (*40*). Parallels between normal and cancer stem cells highlight the importance of understanding normal stem cell biology in the mammary gland to direct studies of breast cancer stem cell biology. Although ß-catenin is not known as an oncogenic factor by itself, several studies have shown that ß-catenin mutation plays a critical role in carcinogenesis by cooperating with other oncogenes, such as Ras and Notch, by inducing a clonal expansion of hyperplastic cells {Harada, 2004 #12; Ayyanan, 2006 #13. These studies suggest that ß-catenin may contribute to the expansion of oncogenic progenitors that are the ultimate targets of cancer therapy. It may be possible to sensitize putative breast tumor stem cells to chemotherapeutic agents by inhibiting this pathway.

Our studies, and others, suggest that conventional cancer therapies that target proliferating, terminally differentiated cells with limited replicative potential may initially lead to a favorable clinical response but will fail to eliminate the small population of cancer stem cells that underpin recurrence. Thus, investigating the mechanisms and signaling pathways that support stem cell maintenance in normal and malignant tissue may provide new targets for therapies designed to complement existing approaches and reduce tumor recurrence.

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Figure Legends

Figure 1 (a.) – (e.) Recombination analysis by PCR. (a.) MECs from compound Catnb$^{lox(ex3)}$/R26R mice (DF) and R26R only (FR) mice were plated and transduced with Ad-Cre1. To detect Cre-mediated recombination, the MECs were fixed and stained for ß-galactosidase expression, magnification 10X. (b.) PCR analysis for Cre-mediated recombination of the Catnb$^{lox(ex3)}$ allele in MECs. + Ad-Cre1: lane 1; –Ad-Cre-1: lane 2. Arrow indicates the PCR product from the recombined allele. (c.) RT-PCR analysis for Cre-mediated recombination. 1 Kb ladder: lane1; compound Catnblox(ex3)/R26R (DF), +RT, lane 2; –RT control, lane 3; R26R (FR), +RT, lane 3; –RT control, lane 4. Arrow indicates the PCR product from the recombined cDNA. Quantitative real time PCR analysis of ß-catenin target genes (d.) cyclin D1 and (e.) ITF2 (average of 2 experiments).

Figure 2 (a.) – (j.) Ad-Cre1 transduced primary mammary epithelial cell outgrowth. (a.) Catnb$^{lox(ex3)}$ MECs were transduced with Ad-Cre1 (+Ad-Cre1) or Ad-ß-gal (–Ad-Cre1) at MOI of 50. 1X10^6 MECs were transplanted into each cleared fat pad. The transplants were removed 8 weeks later and whole-mounted using Carmine Alum, magnification 20X. (b.) DNA recombination in the transplanted tissue was performed using PCR. 1Kb ladder, lane 1; DNA from control transplant, lane 2; DNA from Catnb$^{lox(ex3)}$ transplant, lane 3. Arrow indicates recombination of the Catnb$^{lox(ex3)}$ allele in the transplanted tissue. Zero out of 7 of the Ad-Cre1 transduced transplants grew out, and 7 out of 7 of the Ad-ß-gal (control) transduced transplants grew out. Hematoxylin and Eosin-stained sections of Ad-Cre1 transduced (c.), (d.) and control (e.), (f.) outgrowths. Scale bar = 100 μm. Sections from Ad-Cre1 transduced (g.) and control (i.) outgrowths were stained with Ki67 antibody. Sections from Ad-Cre1 transduced (h.) and control (j.) outgrowths were stained with cyclin D1 antibody. Scale bar = 50 μm.

Figure 3. Whole-mount analysis of timed transplants at 2, 3, and 5 weeks. Catnb$^{lox(ex3)}$ MECs were transduced with Ad-Cre1 or Ad-ß-gal (control) and transplanted into cleared fat pads. Outgrowths were examined at 2 weeks, + Ad-Cre1 (a.), –Ad-Cre1 (b.); 3 weeks, + Ad-Cre1 (c.), –Ad-Cre1 (d.); 5 weeks, + Ad-Cre1 (e.), –Ad-Cre1 (f.), magnification 10X. Representative images of 3 sets of transplants.

Figure 4. CDßgeo cells can self-renew in mammosphere assay and express putative stem cell marker. (a) Bright field. ~1 in 600-1000 CDßgeo cells form a mammosphere. Scale bar = 100 μm (b) CDßgeo mammospheres are cyto-spun onto glass slides, and immunostained for CD49f (red). Nuclei are visualized with DAPI (blue). Scale bar = 20 μm (c) CDßgeo mammospheres are immunostained for TIE-2. Nuclei are visualized with DAPI (blue). Scale bar = 20 μm (d) CDßgeo mammospheres express K14 (red). Nuclei are visualized with DAPI (blue). Scale bar = 50 μm. (e) CDßgeo mammospheres express Keratin 6 (green). Nuclei are visualized with DAPI (blue). Scale bar = 50 μm (f.) Stabilized ß-catenin expression enriches for stem/progenitor cells by increasing the number of mammospheres as well as promoting mammary outgrowths from transplanted

CDßgeo cells. CDßgeo cells transduced with GFP (control), ß-catenin, or ß-engrailed were FACS sorted into $Sca1^+$ and $Sca1^-$ populations and grown in suspension at a density of 20,000 cells per well for 14 days. The mammospheres were passaged once after 7 days, and the passage 2 mammospheres were counted using a Leica dissecting scope. GFP control $Sca1^+$ vs. $Sca1^-$ 1.9 -fold, *$p < 0.02$; GFP $Sca1^+$ vs. ß-catenin $Sca1^+$, 1.6- fold increase, **$p < 0.008$. The efficiency of mammosphere formation is calculated as the number of mammospheres per the number of seeded cells. Data was collected from three individual experiments performed in triplicate sets.

Figure 5. CD ß-geo cells transduced with ß-galactosidase (control), ß-catenin, or ß-engrailed were transplanted into cleared mammary fat pads of Balb/c mice. Outgrowths were taken from two glands per group, stained for whole mounts with Carmine Alum (top, scale bar = 5 mm.) and stained for pathology with hemotoxylin and eosin (bottom, scale bar = 50μm).

Figure 6. Primary mammary epithelial cells were isolated from Balb/c female mice. The cells were immunostained with conjugated antibodies against (a.) CD44, CD24, and (b.) cKIT and SCA, followed by flow cytometry analysis.

Figure 7. Clinically relevant doses of radiation increase the percentage of progenitor cells (assayed as % side-population (%SP)) and increase the percentage of Sca1+ progenitors cells in the side population. (A) Mammary epithelial cells were isolated from Balb/c mice, cultured 3 days, irradiated, and analyzed for %side population by Hoechst 33342 staining/flow cytometry. Radiation selectively increases the progenitor fraction (%SP). $P = 0.015$ (2 Gy), 0.008 (4 Gy), 0.05 (6 Gy), two-tailed T-test. (B) Cells were analyzed for Sca-1 positivity within the side population by flow cytometry 24 hours after irradiation. Radiation selectively increased the Sca1+ (progenitor) fraction within the side population by killing the more sensitive Sca1- (non-progenitor) cells. $P < 0.05$ comparing $Sca1^+$ to $Sca1^-$ at 0 vs. 2 –8 Gy. 2Gy vs. higher doses not significant.

Figure 8. Radiation induces more DNA damage foci in $Sca1^-$ cells 2 hours after irradiation. (a.) – (d.) $Sca1^+$ and $Sca1^-$ cells from Balb/c MECs were sorted onto glass slides following radiation at 2 Gy, immunostained with anti-phospho-H2AX (scale bar: 10um). (e.) There were significantly more DNA-damage foci in the Sca1- population compared to $Sca1^+$ cells ($Sca1^+$ vs $Sca1^-$ 3.7 fold difference, $p < 0.05$). (f.) More Sca^- cells display DNA-damage foci compared to Sca^+ cells.

Figure 9. $Sca1^+$ and $Sca1^-$ cells have distinct cell cycle profiles. Primary Balb/c MECs were sorted into Sca^+ and Sca^- populations. Cells were stained with 7AAD and Pyronin Y to distinguish between G0 and G1, respectively. While $Sca1^-$ cells exhibit no redistribution in response to radiation, both the G0 and S/G2/M populations among $Sca1^+$ cells increase after radiation.

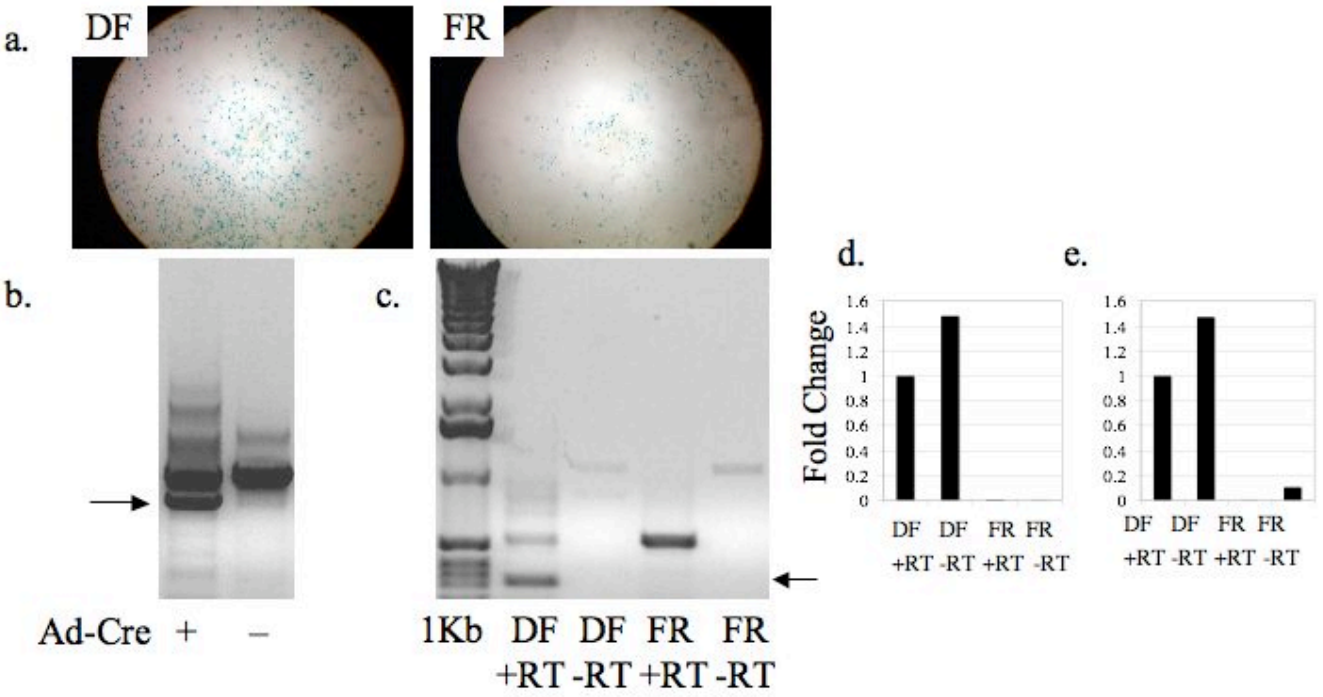
Figure 10. Radiation (2 Gy, the daily dose of radiation typically used in the clinic setting) does not decrease the number of $Sca1^+$ colonies. Primary mouse mammary epithelial cells were dissected from 8-week-old Balb/c mice and cultured for 3 days. Cultures were irradiated with 0 or 2 Gy, incubated with Sca1 antibody, and sorted onto Matrigel by flow cytometry. Irradiated and unirradiated $Sca1^+$ and $Sca1^-$ cells were allowed to grow for 2 weeks and scored for colony formation by 2 independent investigators.

Figure 11. Radiation selectively activates ß-catenin and Survivin in $Sca1^+$ cells. (a.) Quantitative assessment of activated ß-catenin signaling was assessed by flow cytometry after staining for Sca1 and unphosphorylated ß-catenin. (b.) Real-time PCR for survivin expression was performed 24 hours after irradiation in $Sca1^+$ and $Sca1^-$ cells.

Figure 12. Differences in ß-catenin level and localization between $Sca1^+$ and $Sca1^-$ subpopulations. (a) $Sca1^+$ and $Sca1^-$ subpopulations were stained with non-phosphorylated ß-catenin and analyzed by flow cytometry with an Alexa-488 antibody against ß-catenin. ~95% of the $Sca1^+$ cells contain non-phosphorylated ß-catenin, while only ~1.2% of the Sca1- cells contain non-phosphorylated ß-catenin. (b) Immunostaining for non-phosphorylated ß-catenin in $Sca1^+$ and $Sca1^-$ cells at 0 Gy (sham irradiation) and 4 Gy. ß-catenin is visualized in green, and the nuclei are stained with DAPI in blue. Scale bar = 5 μm. Images were captured by deconvolution microscopy using a Zeiss AxioVert S100 TV microscope and a DeltaVision restoration microscopy system (Applied Precision, Inc.). For high-resolution deconvolved images, captured raw images were deconvolved with the DeltaVision constrained iterative algorithm.

Figure 13. Anesthetized Balb/c mice were immobilized supine and mammary glands (entire ventral surface) were irradiated. MECs were isolated 48 h after irradiation and analyzed immediately for Sca1, and CD24/CD29 by flow cytometry. (a.) Radiation selectively increased the $Sca1^+$ (progenitor) fraction and decreased the $Sca1^-$ (nonprogenitor) cells. *$p < 0.0001$. In vivo radiation increased the percentage of $CD24^+CD29^+$ positive cells from MCF-7 cells but not uncultured MECs. (b.) Freshly digested MECs were analyzed for $lin^- CD24^+CD29^+$ 48 h after in vivo irradiation. (c.) The $CD24^+CD29^+$ population is sensitive to radiation. (d.) Radiation selectively decreased the $lin\text{-}CD24^+CD29^{lo}$ fraction cells ($p = 0.003$ 0 Gy vs. 2 Gy and $p = 0.0002$ 0 Gy vs 4 Gy.

Figure 1.


a.
DF
FR
b.
c.
d.
e.
Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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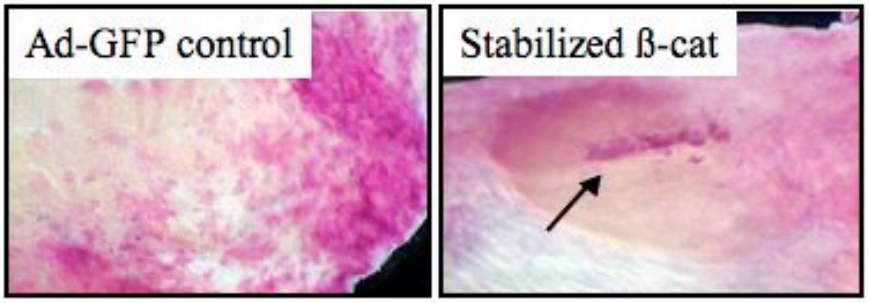
DF +RT
DF -RT
FR +RT
FR -RT
Ad-Cre + –
1Kb DF +RT DF -RT FR +RT FR -RT

Figure 2.

a.



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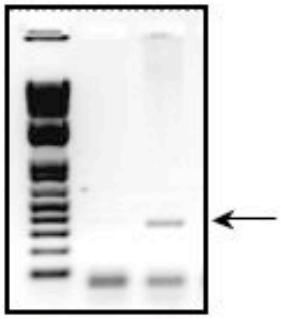


	# Outgrowth
Stabilized ß-catenin	0/7
Ad-GFP control	7/7

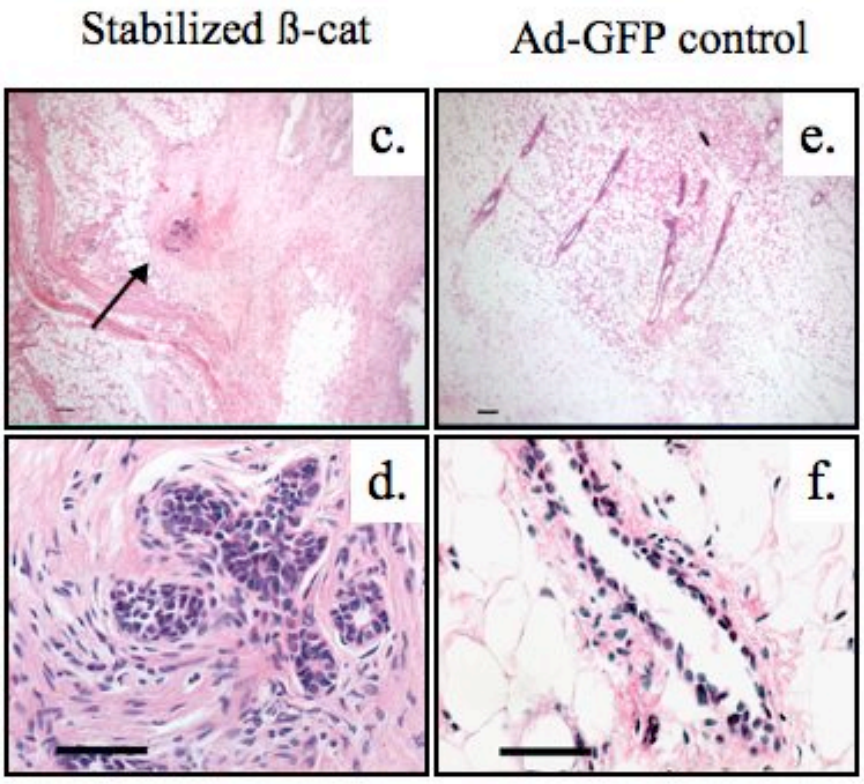
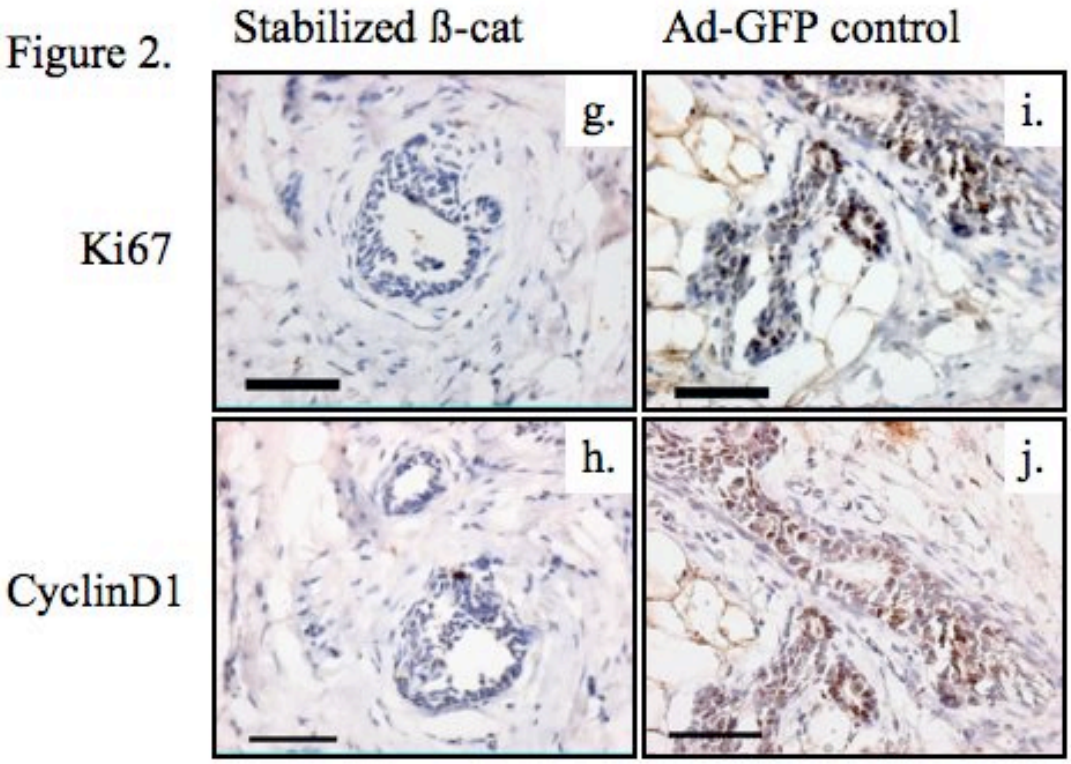


Figure 2.



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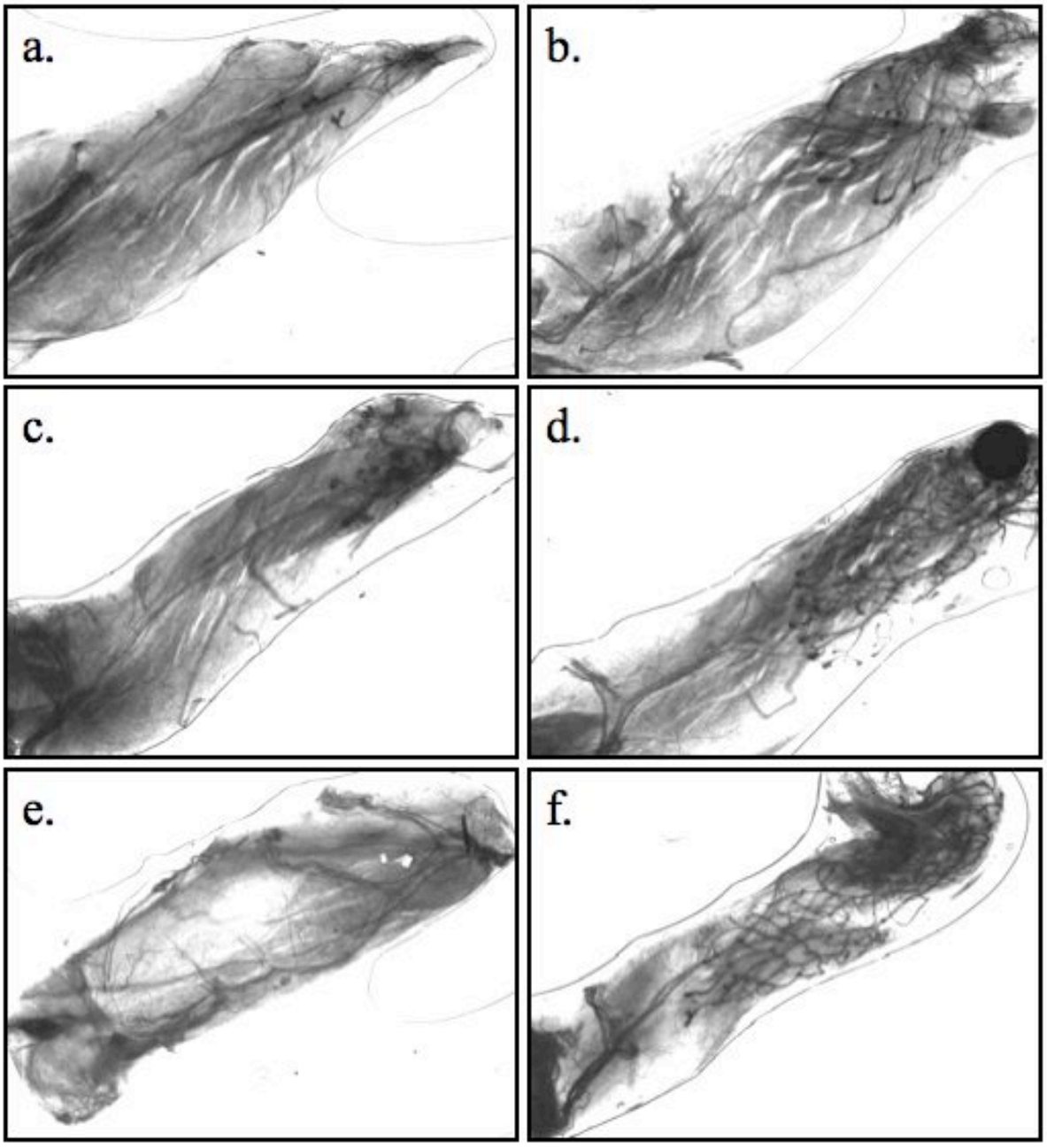


Figure 4..

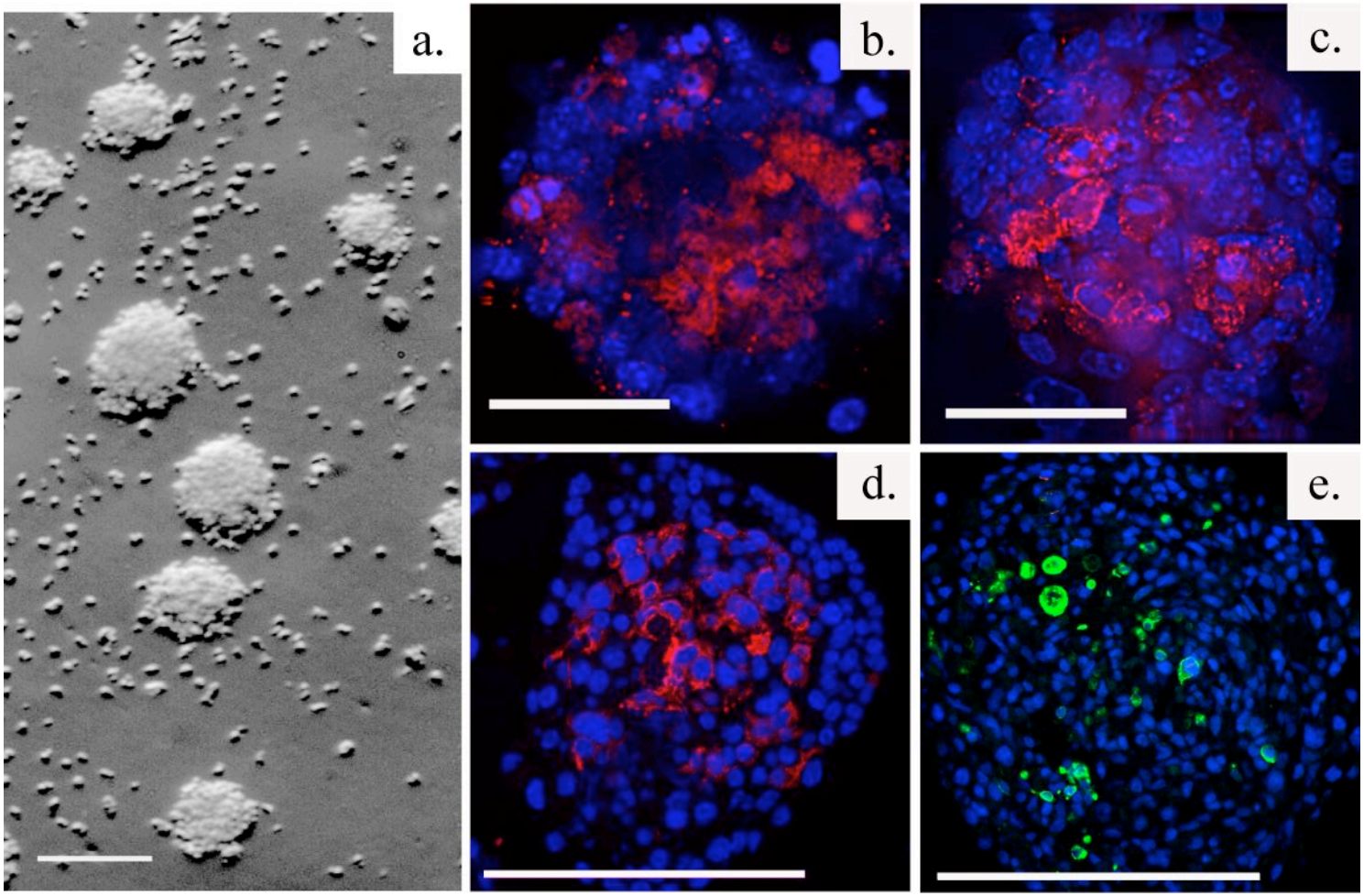


Figure 4.

f.

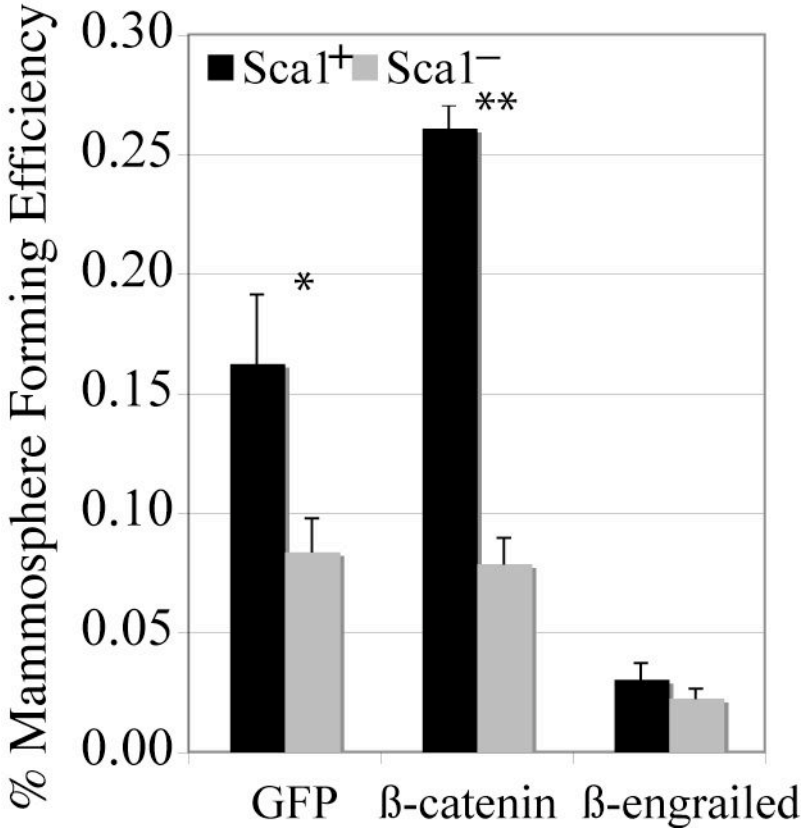

% Mammosphere Forming Efficiency
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Sca1+ Sca1−
*
**
GFP
ß-catenin
ß-engrailed

Figure 5.

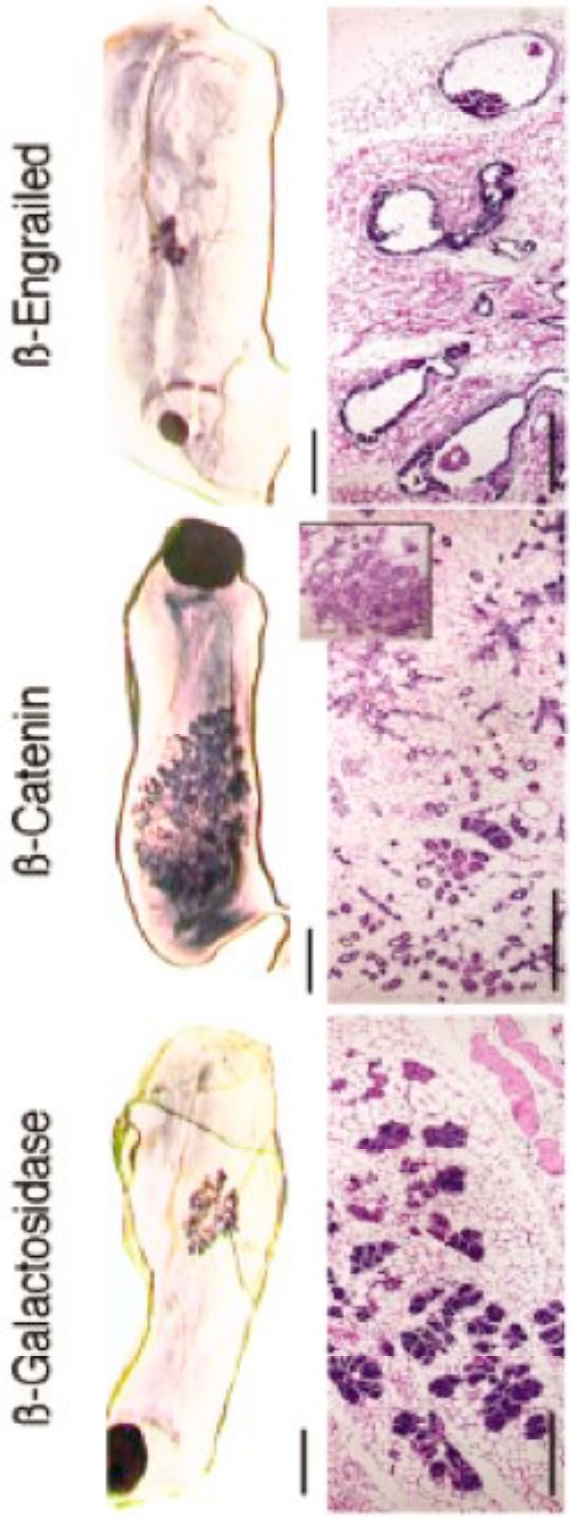
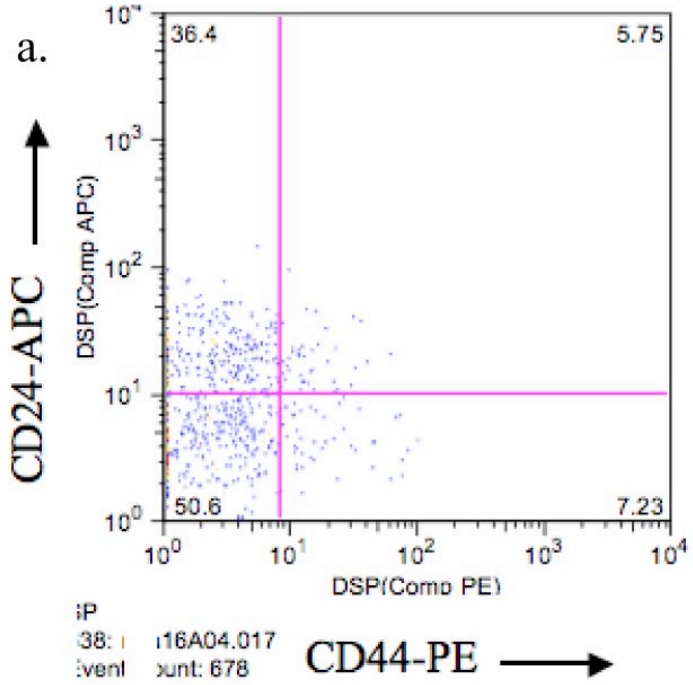


Figure 6.



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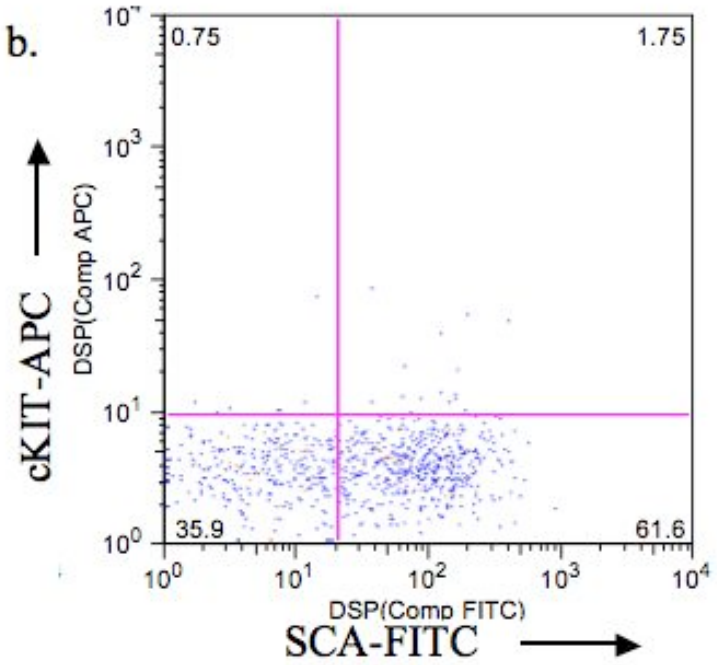

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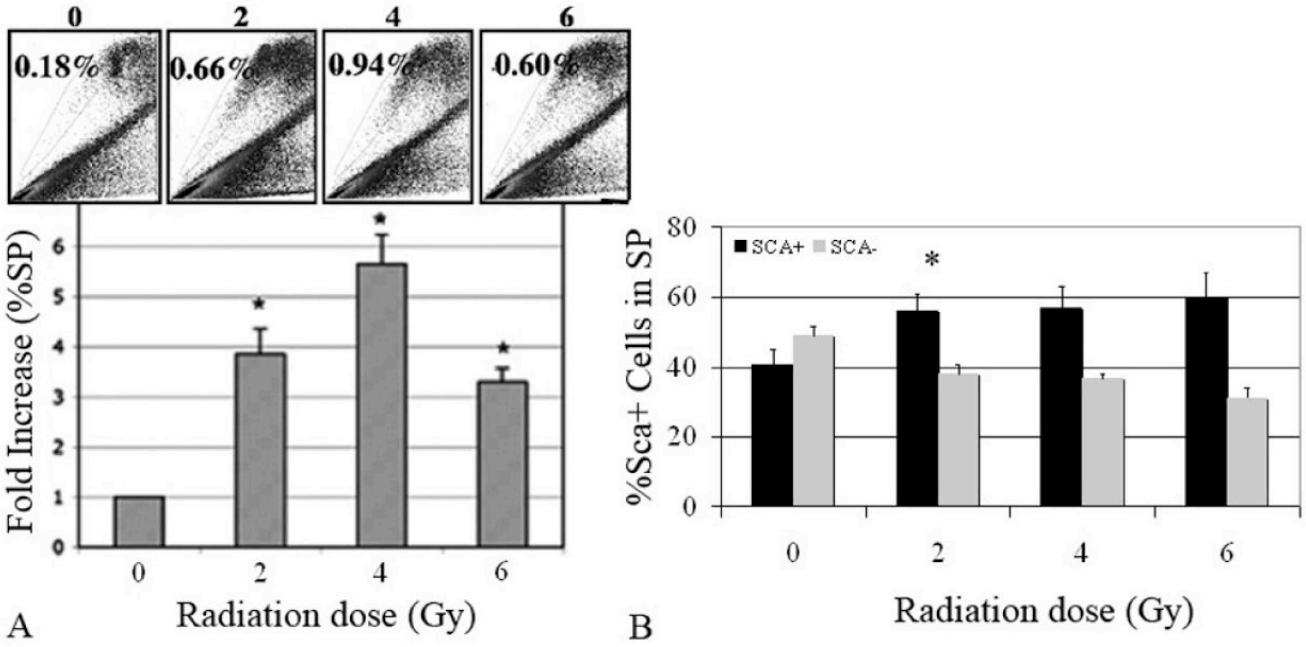
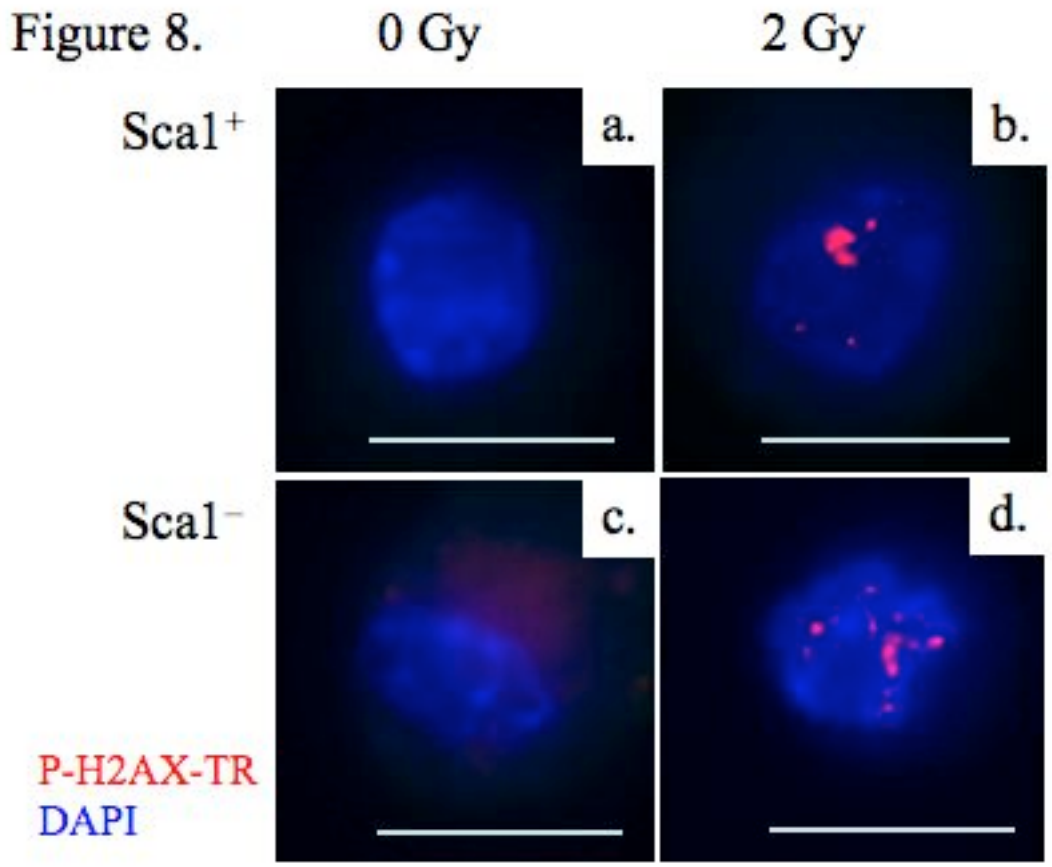
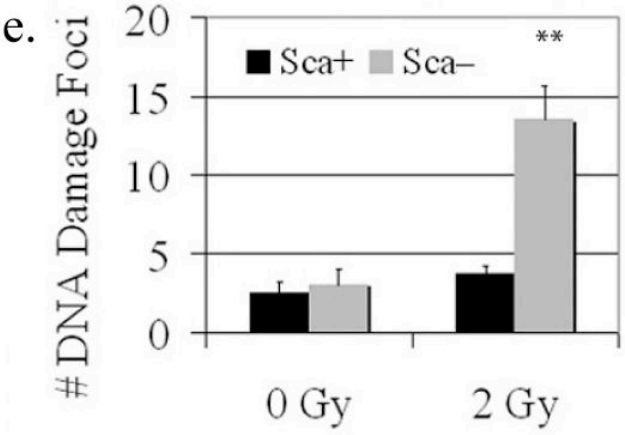

0
2
4
6
0.18%
0.66%
0.94%
0.60%
Fold Increase (%SP)
0
1
2
3
4
5
6
0
2
4
6
Radiation dose (Gy)
A
%Sca+ Cells in SP
SCA+
SCA-
*
0
20
40
60
80
0
2
4
6
Radiation dose (Gy)
B

Figure 8.


0 Gy
2 Gy
Sca1+
Sca1−
a.
b.
c.
d.
P-H2AX-TR
DAPI

e.


DNA Damage Foci
20
15
10
5
0
Sca+
Sca–
**
0 Gy
2 Gy

f.

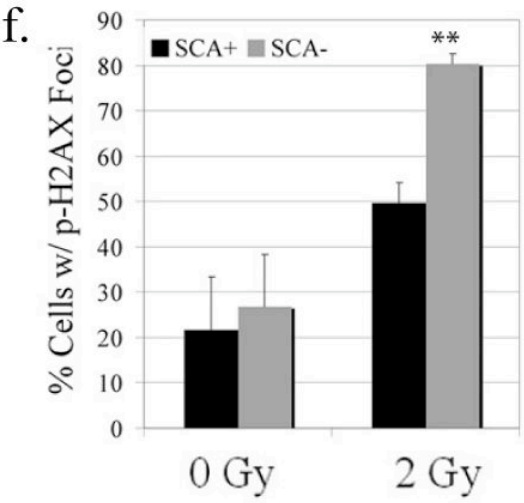
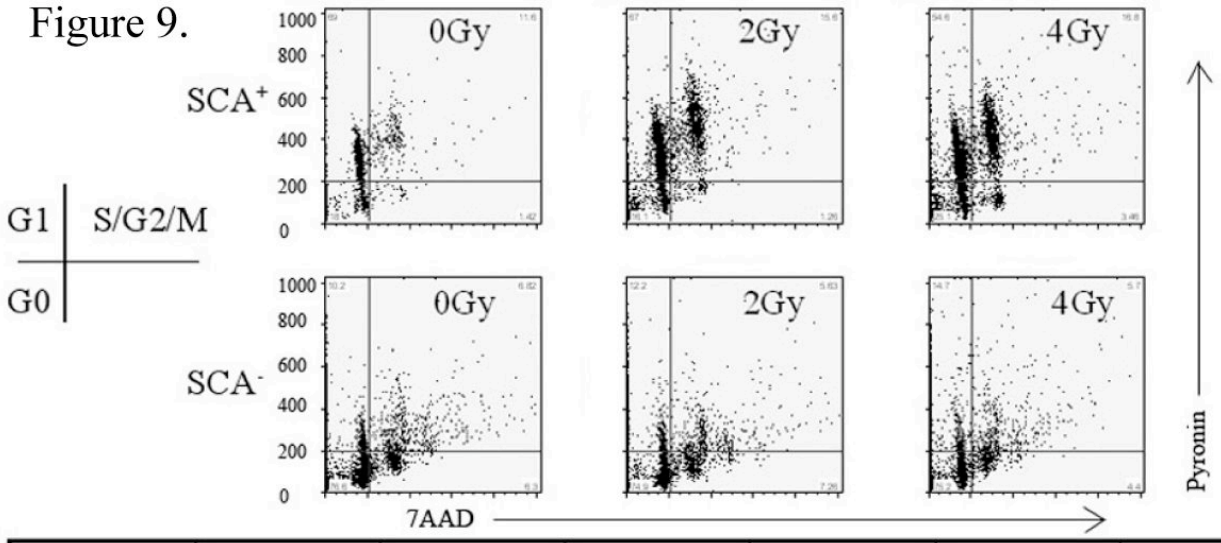

% Cells w/ p-H2AX Foci
90
80
70
60
50
40
30
20
10
0
SCA+
SCA-
**
0 Gy
2 Gy

Figure 9.



	0 Gy Sca+	2 Gv Sca+	4 Gy Sca+	0 Gy Sca-	2 Gy Sca-	4 Gy Sca-
G0	18	16.1	25.1	76.6	74.9	75.2
G1	69	67	54.6	10.2	12.2	14.7
S/G2/M	11.6	15.6	16.8	6.82	5.63	5.7

Figure 10.

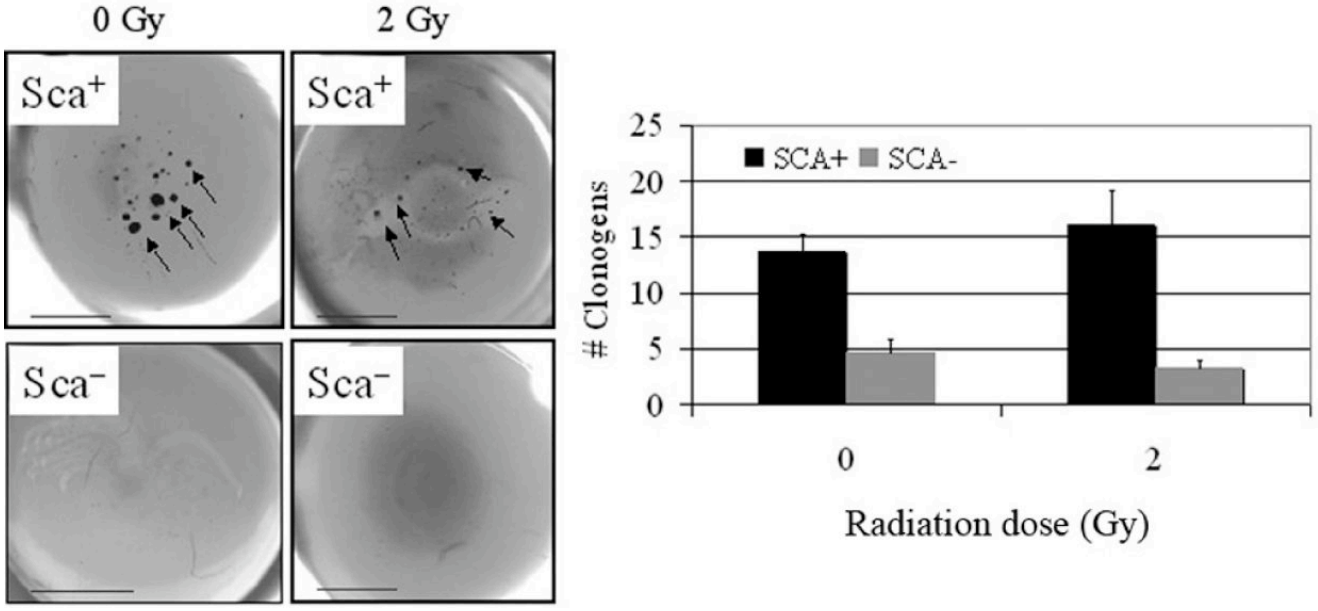


Figure 11.

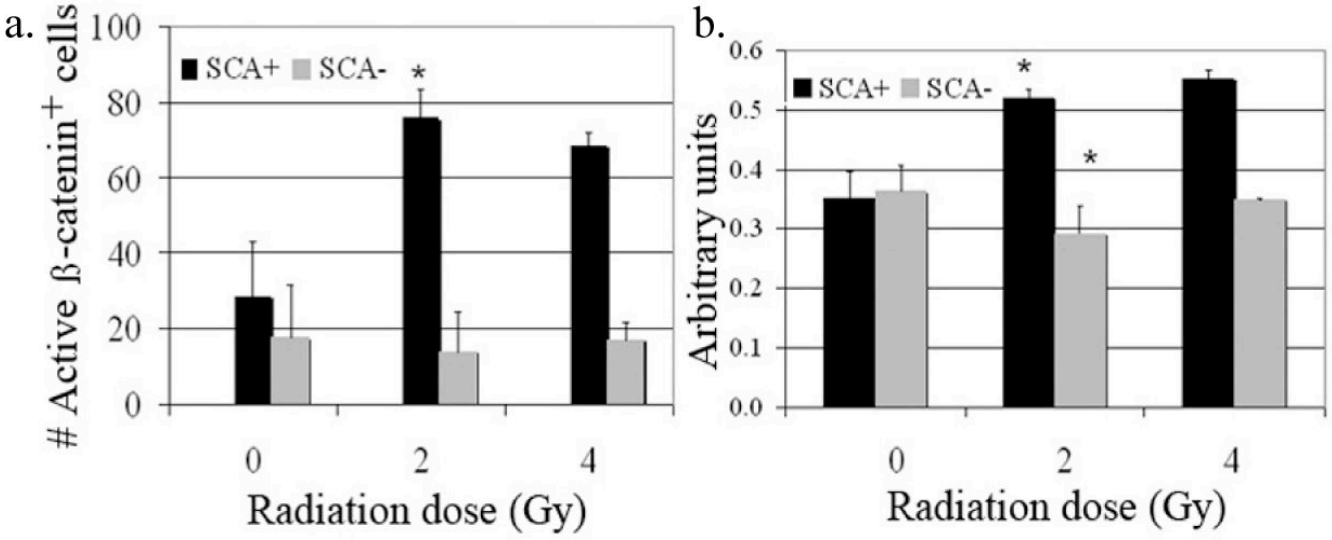

a.
Active ß-catenin+ cells
100
80
60
40
20
0
SCA+
SCA-
*
0
2
4
Radiation dose (Gy)
b.
Arbitrary units
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SCA+
SCA-
*
*
0
2
4
Radiation dose (Gy)

Figure 12.

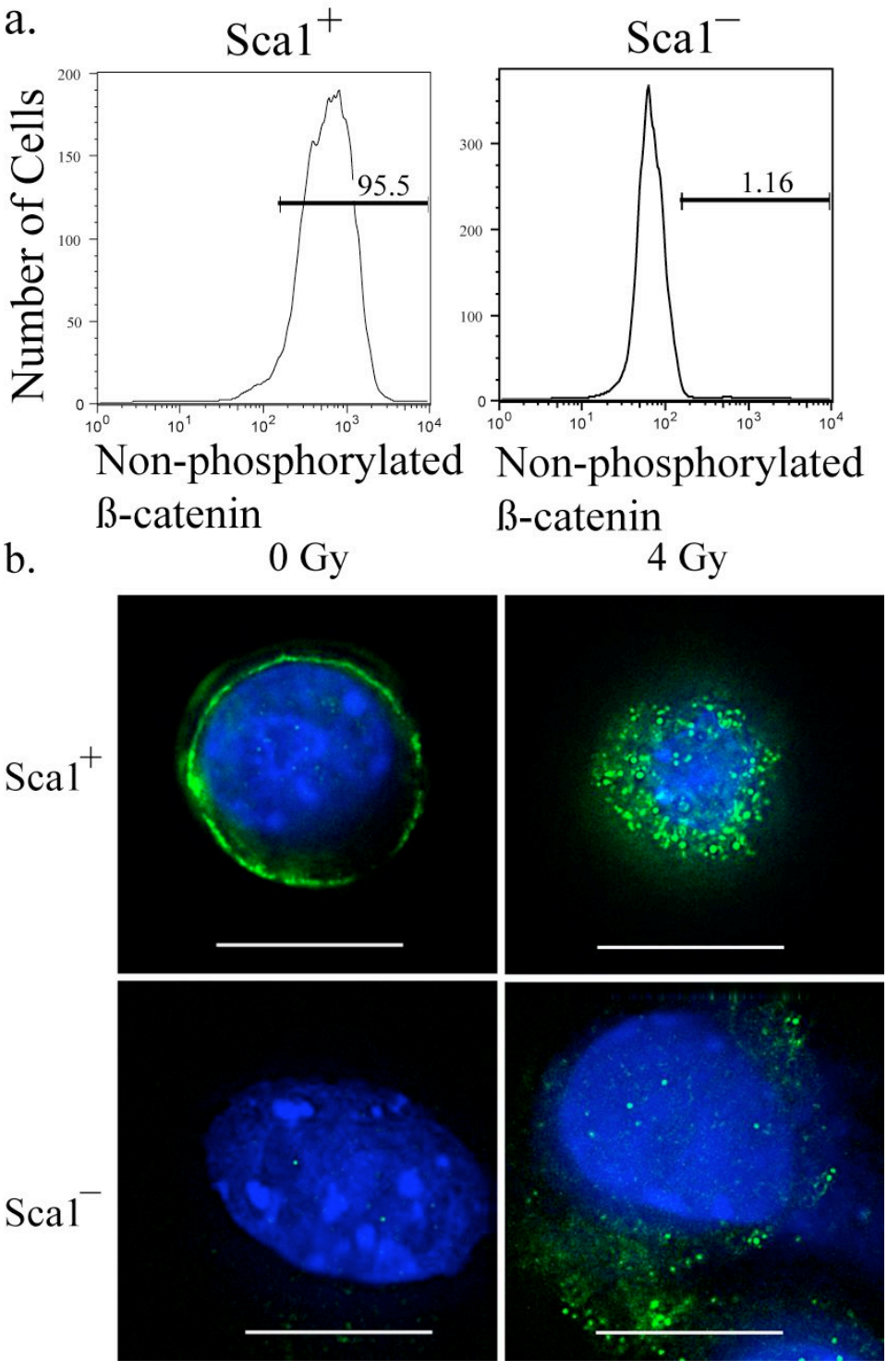
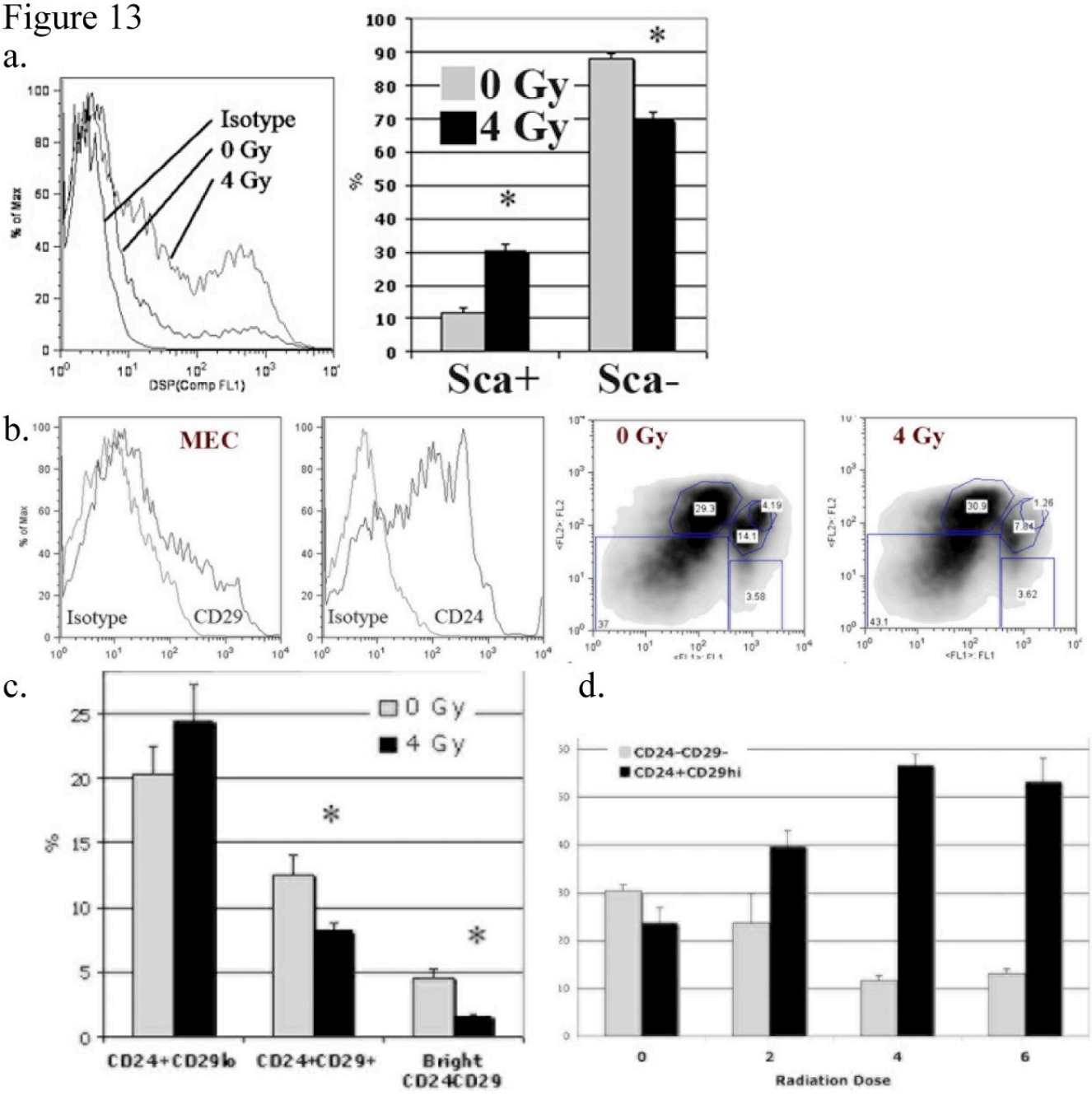

a.
Sca1+
Sca1−
Number of Cells
95.5
1.16
Non-phosphorylated ß-catenin
Non-phosphorylated ß-catenin
b.
0 Gy
4 Gy
Sca1+
Sca1−

Figure 13


a.
Isotype
0 Gy
4 Gy
% of Max
DSP(Comp FL1)
0 Gy
4 Gy
%
Sca+
Sca-
b.
MEC
Isotype
CD29
Isotype
CD24
0 Gy
4 Gy
29.3
4.19
14.1
3.58
37
30.9
1.26
7.84
3.62
43.1
c.
0 Gy
4 Gy
%
CD24+CD29lo
CD24+CD29+
Bright CD24CD29
d.
CD24-CD29-
CD24+CD29hi
Radiation Dose

Appendices

Appendix 1

- **Mercy S. Chen***, Wendy A. Woodward*, Fariba Behbod, Maria P. Alfaro, Thomas Buchholz, and Jeffrey M. Rosen. Wnt/ß-catenin-Mediated Radiation Resistance of Mouse Mammary Stem-like/Progenitor Cells (Submitted).

  *These authors contributed equally

Appendix 2

- **Mercy S. Chen***, Wendy A. Woodward*, Fariba Behbod, Maria P. Alfaro, and Jeffrey M. Rosen. Wnt/ß-Catenin Mediates Radiation Resistance of Stem Cell Antigen-1 Positive Progenitors in an Immortalized Mammary Gland Cell Line (Submitted).

Appendix 3

- **Mercy S. Chen***, Wendy A. Woodward*, Fariba Behbod, Jeffrey M. Rosen. On mammary stem cells. J. Cell Science 2005 Aug 15;118(Pt 16):3585-94.

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WNT/ß–catenin mediates radiation resistance of mouse mammary progenitor cells

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Classification: Biological Sciences, Medical Sciences

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Nonstandard abbreviations used: MECs, mammary epithelial cells; LRCs, label-retaining cells; SP, side population; $Sca1^{+}$, stem cell antigen-1; BCRP1, breast cancer resistance protein-1; Gy, gray (1 Gy = 100 rads).

Conflict of interest: The authors declare that no conflict of interest exists.

ABSTRACT

Recent studies have identified a subpopulation of highly tumorigenic cells with stem/progenitor cell properties from human breast cancers, and it has been suggested that stem/progenitor cells, which remain after breast cancer therapy, may give rise to recurrent disease. We hypothesized that progenitor cells are resistant to radiation, a component of conventional breast cancer therapy, and that that resistance is mediated at least in part by Wnt signaling, which has been implicated in stem cell survival. To test this hypothesis, we investigated radioresistance by treating primary Balb/c mouse mammary epithelial cells with clinically relevant doses of radiation and found enrichment in normal progenitor cells (stem cell antigen-1-positive and side population progenitors). Radiation selectively enriched for progenitors in mammary epithelial cells isolated from transgenic mice with activated Wnt/ß-catenin signaling but not for background-matched controls, and irradiated stem cell antigen-1-positive cells had a selective increase in active ß-catenin and survivin expression compared with stem cell antigen-1-negative cells. In clonogenic assays, colony formation in the stem cell antigen-1-positive progenitors was unaffected by clinically relevant doses of radiation. In the human breast cancer cell line MCF-7, radiation induced enrichment of side population progenitors as well as enrichment of the $Lin^{-}CD24^{+}CD29^{+}$phenotype observed previously for stem cells in the normal mammary gland. These data demonstrate that compared with differentiated cells, progenitor cells have different cell survival properties which may facilitate the development of targeted anti-progenitor cell therapies.

INTRODUCTION

It has been speculated that stem cells may represent the cellular origins of cancer, because they exist quiescently for long periods of time, and could, accumulate multiple mutations over the life-span of an organism ultimately giving rise to tumors when stimulated to proliferate (1). Recently, it was reported that highly tumorigenic cells with properties consistent with those of stem/progenitor cells can be isolated from human breast cancers (2). These data suggest that cancer stem cells may exist in human breast cancer, and that they may have different biologic features than other, more differentiated cells that constitute the majority of the cells in human breast cancers. Conceivably, cancer stem cells may be more resistant to conventional breast cancer therapies, which may ultimately result in recurrence or metastasis even when remarkable initial responses are observed clinically (3).

Although the elucidation of sensitive stem and progenitor cell markers in the mammary gland and in breast tumors has been relatively recent, the normal tissue response of stem and progenitor cells to radiation, an integral component of multidisciplinary breast cancer therapy, has been the subject of several decades of radiobiological data. The development of a simple, reproducible *in vivo* clonogenic assay in the jejunum, where the effects of radiation on stem cells can be easily measured, led to the conclusions that in the small intestine, the cells at position 4-5 in the crypt now identified as *the* stem cells are exquisitely sensitive to radiation (4). However, a second population of *potential* stem cells, elsewhere called transiently amplifying cells or progenitors, exists that can be called into action in the event of lethal damage to the stem cells. At low doses of radiation, the crypt of the small intestine contains 4-5 clonogenic

regenerating cells, but at higher doses of radiation up to 30-40 potential clonogenic regenerators can be called into play (4). The elucidation of stem/progenitor cell markers in the mammary gland now allow this work to be tested at the cellular level in the mammary gland. We hypothesize that mammary gland progenitors may be resistant to radiation and that this resistance is mediated by the ß-catenin stem cell survival signaling pathway.

ß-catenin is an essential component of both intercellular junctions and the canonical Wnt signaling pathway, which has been implicated in stem cell survival (16). In recent studies, activation of ß-catenin in granulocyte-macrophage progenitors in chronic myelogenous leukemia appeared to enhance their self-renewal activity and leukemic potential (3). In addition, studies in the intestine and mammary gland have linked ß-catenin signaling to stem cell survival and tumorigenesis (17-19).

Several methods are currently being used to isolate and study mammary stem/ progenitor cells, including long-term bromodeoxyuridine labeling to identify label-retaining cells (LRCs), Hoechst dye efflux to identify side population (SP) properties, and the potential stem/progenitor cell-cell surface markers, such as stem cell antigen-1 (Sca1) and alpha6- and ß1-integrins(5-7). In the hematopoietic system, cells that efflux Hoechst 33342 dye have been shown to comprise a small fraction of bone marrow, which are capable of recapitulating the bone marrow in irradiated mice establishing their functional capacity as hematopoietic stem cells (8). These cells are represented as a side population (SP) on flow cytometry analysis present in both mouse and human mammary glands (9-12). In hematopoietic stem cells, the efflux of Hoechst dye is due to the presence of a family of drug-effluxing protein pumps, including the breast cancer resistance protein-1

(BCRP1)/ABCG2 transporter, that may be responsible for drug resistance in many types of cancer (13). In the mouse mammary gland, the SP phenotype is dependent on several members of the ABC transporter family (14). Outgrowth experiments using SP are confounded by the toxicity of the Hoechst dye (10), however the SP fraction in mouse mammary gland is enriched for long-term bromodeoxyuridine LRCs, as well as Sca-1 positive cells, which have the capacity to generate functional mammary outgrowths in transplantation experiments (9). Shackleton et al have recently confirmed outgrowth potential of a Sca-1^+ population, but demonstrated that the majority of outgrowth potential of Sca-1^+ population in fact lies in the small, Sca-lo positive cells (6). Taken together, these data support the SP and Sca-1^+ phenotypes as useful surrogates for stem-like/progenitor cells. A thoughtful review of the mammary SP phenotype was published recently (15), and review of the literature regarding both SP and Sca-1 suggests that each of these represent markers useful for isolating potential downstream progenitors (5) rather than the more primitive stem cells isolated as described by Shackleton et al as lin^- $CD24^+CD29^+$ (6).

Although it has been speculated that stem or progenitor cells in the mammary gland are more resistant to conventional cancer therapies, this relationship has not been explicitly demonstrated. Here we demonstrate that progenitors in murine MEC culture are enriched by clinically relevant doses of ionizing radiation and this enrichment is enhanced by ß-catenin stabilization. In human breast cancer MCF-7 cells, radiation induced enrichment of both SP progenitors and the $Lin^-CD24^+CD29^+$ population. These findings from normal and genetically manipulated mouse mammary glands may have

important implications for the evaluation of new and current cancer therapies and the development of future new anti-stem cell-targeted therapies.

RESULTS

Primary MEC progenitor cells are radioresistant. To test our hypothesis that progenitor cells in the mammary gland are resistant to radiation compared to the non-progenitors, cultured primary mammary epithelial cells (MECs) from Balb/c mice were irradiated and the percentage of SP progenitor cells in the total population after treatment was analyzed by flow cytometry. MECs were isolated, cultured for 72 h and irradiated 24 h prior to analysis. Irradiation of Balb/c MECs lead to a 4-fold increase in the % SP cells at 2 Gy to a nearly 6-fold increase at 4 Gy. The increase in %SP decreased at 6 Gy, but was still more than 3-fold higher than baseline (Figure 1a). Irradiation of sorted NSP cells does not lead to SP cells suggesting that this increase in the SP fraction is not a result of radiation induction of BCRP in MECs (data not shown). To further explore the potential clinical relevance of these findings we examined the human breast cancer cell line MCF-7, which has been reported previously to contain a subpopulation with stem/progenitor characteristics (20, 21). The %SP in MCF-7 cells was significantly increased by radiation (0.08, 0 Gy vs 0.19, 4 Gy; $p = 0.05$ (Figure 1b).

Welm et al (9) showed that the SP population is enriched for $Sca1^+$ cells and that $Sca1^+$ progenitors give rise to outgrowths when transplanted. In our study, the percentage of $Sca1^+$ cells within the SP increased with radiation while the percentage of $Sca1^-$ cells is selectively decreased with radiation (Figure 1c). Stingl et al recently reported very high levels of $Sca\text{-}1^+$ cells after culture of primary MECs similar to the methods used in this study and as opposed to analysis of freshly isolated MECs (7). To evaluate the impact of culturing, we radiated the mammary glands of anesthetized Balb/c mice *in vivo*, dissected the glands and performed Sca-1 analysis on freshly dissociated

MECs (Figure 1d). *In vivo* radiation (4 Gy) significantly decreased the percentage of $Sca1^-$ cells (88% 0 Gy vs 70% 4 Gy $p < 0.0001$) and increased the percentage of $Sca1^+$ cells (12% vs 30%, $p < 0.0001$). The percentage of $Sca1^+$ cells after fresh digestion ranged from 10-25% and consistently increased approximately 3-fold after 4 Gy (n =3).

The $CD24^+CD29^+$ population recently characterized for its ability to give rise to mammary outgrowths from a single cell (6) was examined in parallel using freshly isolated MECs. Using freshly isolated MECs, we observed a level of $CD24^+CD29^+$ cells similar to that published by Shackleton et al (< 10%). *In vivo* radiation (4 Gy) did not enrich for this stem cell population and in fact decreased this population by approximately one-third ($lin^-CD24^+CD29^+$ 12.5% 0 Gy vs. 8% 4Gy, $p = 0.01$, Figure 2a). Radiation decreased the brightest double positive cells in this population by approximately two-thirds ($p = 0.002$, Figure 2a). For comparison, the radiation resistance of this population was also examined in MCF-7 cells. Radiation dramatically increased the $lin^-CD24^+CD29^+$ population in MCF-7 cells (34% 0 Gy vs. 53% 2 Gy and 71% 4 Gy, $p = 0.003$ 0 Gy vs. 2Gy and $p = 0.0002$ 0 Gy vs. 4 Gy, Figure 2b). Both the $CD24^+CD29^{lo}$ and double negative populations were significantly diminished after irradiation.

Since radiation cell killing is generally attributed to mitotic cell death, clonogenic assays to assess the replicative competence of radiated subpopulations were performed. $Sca1^-$ cells generally failed to form colonies when sorted into Matrigel and allowed to grow for two weeks in standard radiation clonogenic assays, while $Sca1^+$ cells readily formed colonies. Irradiating cells (2 Gy) prior to clonogenic assays yielded no reduction in the number of clonogens (Supplemental Figure 1). To substantiate that the $Sca1^+$ cells

are cycling, we performed cell cycle analysis following radiation treatment. Primary Balb/c MECs were sorted into $Sca1^+$ and $Sca1^-$ populations and stained with 7ADD and pyronin Y to distinguish between G0 and G1 (Supplemental Figure 2). At baseline both populations contain a significant cycling population ($Sca1^+$ 11.6% and $Sca1^-$ 6.82%) but inversely related G0 and G1 populations ($Sca1^+$, G0 = 18% and G1 = 69%; $Sca1^-$, G0 = 76.6% and G1 = 12.2%). While $Sca1^-$ cells exhibited no redistribution in response to radiation treatment, both the G0 and S/G2/M populations among $Sca1^+$ cells increased after radiation treatment.

Because double strand DNA breaks lead to lethal radiation damage more often than single strand breaks (22), we examined radiation induced DNA double strand breaks using immunofluorescent staining with a γ-phospho-H2AX antibody. This antibody binds to DNA flanking the double strand breaks creating discrete foci. Phosphorylation of the histone variant H2AX at the site of DNA damage occurs rapidly after ionizing radiation, and the formation of DNA damage foci is ATM-dependent (23). Two h after radiation treatment, significantly more $Sca1^-$ cells contained foci and there were more foci per damaged cell in $Sca1^-$ cells (Figure 3).

Wnt/ß-catenin signaling mediates progenitor cell resistance. To determine the role of the putative mammary stem cell survival factor Wnt/ß-catenin in mediating radioresistance of SP cells, MECs were isolated from mouse mammary tumor virus (MMTV)-driven Wnt-1 transgenic mice at 10-12 weeks of age and expanded in tissue culture for 72 h. Cells were then irradiated and stained with Hoechst 33342 for SP analysis 24 h later. MECs isolated from Wnt-1-induced hyperplasias exhibited a trend

toward higher radiation-induced increase in %SP than MECs isolated from mice of a matched background ($p = 0.08$, Figure 4a). Consistent with data reported by Liu et. al. (24), the %SP in MECs from Wnt-induced hyperplasias was significantly higher (> 2 fold) than those in background-matched controls (0.75 vs. 0.32, respectively, $p < 0.05$). The radiation-induced increase in the %SP in the wildtype FVB mice was less remarkable than that observed in MECs from Balb/c mice, consistent with previous studies demonstrating marked differences in radiation response between mouse strains including increased radiation-induced genomic instability and increased susceptibility to radiation induced mammary epithelial tumors in Balb/c mice due to a functional polymorphism (25, 26).

The effect of increased Wnt signaling on SP cell radioresistance was also determined using MECs from C57BL/6 transgenic mice that contained a floxed allele of ß-catenin exon III (27) isolated and transduced with an adenovirus encoding Cre recombinase (AdCre) or a comparable titer of a control adenovirus encoding ß-galactosidase (AdLacZ) on day 3 of culture. Cells were irradiated on day 4 and stained with Hoechst 33342 for SP analysis 24 h later. PCR was used to demonstrate efficient recombination (>75%) in the primary MEC cultures transduced with AdCre (data not shown). Primary MECs containing stabilized ß-catenin exhibited a higher radiation-induced increase in %SP than AdLacZ treated controls (0 vs. 2 Gy $p < 0.05$, 0 vs. 4 Gy $p < 0.05$) (Figure 4a). The lack of radiation-induced change in %SP in the C57Bl/6 MECs compared to the Balb/c MECs may be due to strain differences in mice.

We examined the role of Wnt/ ß-catenin signaling in response to radiation in wild-type MECs from Balb/c mice with flow cytometry on fixed cells. Staining with the

anti-non-phospho- ß-catenin-PE antibody that binds to activated ß-catenin showed that ß-catenin is selectively activated in $Sca1^+$ cells in response to radiation, whereas ß-cat staining in $Sca1^-$ cells is unchanged in response to radiation (Figure 4b). Survivin, a bifunctional member of the inhibitor of apoptosis gene family, has been shown to be upregulated by TCF/ß-catenin in intestinal progenitor cells upon UV-B irradiation (28). In addition, survivin has been shown to play an essential role in mitosis, in both the segregation of sister chromatids and the assembly stabilization of microtubules in late mitosis (29). This suggests that overexpression of ß-catenin may enhance cell survival on radiation treatment at least in part by regulating survivin. Using real-time PCR, we demonstrated that survivin mRNA expression was selectively enhanced in $Sca1^+$ cells in response to radiation ($p = 0.01$; Figure 4b).

DISCUSSION

To our knowledge, this study is the first to demonstrate that progenitor cells in the mammary gland are more resistant to clinically relevant doses of radiation than are non-progenitors, which constitute the bulk of the mammary gland, and that overexpression of the Wnt/ß-catenin pathway can enhance the radioresistance of progenitor cells. In wildtype mice, this effect varies by mouse strain and is most pronounced in the radiosensitive Balb/c strain where enrichment of the progenitor population is statistically significant at 2 Gy. These experiments also demonstrate that radiation resistance in primary MECs can be altered through manipulation of the Wnt/ß-catenin stem cell survival pathway. Understanding the mechanisms of resistance in normal MECs is an important step towards designing novel therapies for tumor progenitor cells.

Hematopoietic SP cells have been shown to possess "stemness" through their ability to recapitulate bone marrow (8). SP cells in the mammary gland represent a heterogeneous population (15) but are enriched for long-term bromodeoxyuridine LRCs as well as cells positive for Sca-1, a putative stem cell marker, which are capable of producing mammary outgrowths after transplantation into cleared murine mammary fat pads (9). Sca-1 cells from the Balb/c-derived Comma-Dβ cell line have been characterized by Deugnier et al and were similarly found to generate *in vivo* outgrowths whereas Sca-1^{lo} cells exhibited decreased outgrowth potential (30). They reported that Sca1+ cells stain for the putative markers phenotypes reported to represent primary mouse stem cells (CD24, alpha 6-integrin) (7) and breast tumor progenitors (CD44)(2).

Selection and identification of putative progenitor markers has been complicated, and few comparisons have been made between different laboratories studying these phenotypes. In contrast to findings reported here and previously from our laboratory regarding the outgrowth potential of $Sca1^{+}$ cells (9), Shackleton et al report that mammary gland repopulating units are best characterized by $CD24^{+}Sca^{lo}CD29^{+}$ and that $Sca1^{hi}$ cells did not display outgrowth potential *in vivo* (6). The population included in the $Sca1^{lo}$ gate described in the Stingl paper would typically be included in the $Sca1^{+}$ population isolated either by magnetic bead sorting or from Sca-EGFP knockin mice by FACS sorting for EGFP used previously and here that have generated outgrowths *in vivo* in our hands. Indeed, Shackleton report a three-fold enrichment in outgrowth potential among $Sca1^{lo}$ vs $Sca1^{hi}$ populations. This suggests that the critical Sca1 population is $Sca1^{lo}$ rather than $Sca1^{hi.}$ Using magnetic bead sorting to distinguish $Sca1^{+}$ and $Sca1^{-}$ isolated from the COMMA-D cell line Deugnier et al also have reported recently that

Sca1^{+} cells also display increased outgrowth potential (30). Similar discrepancies exist regarding the reported outgrowth potential of CD24^{hi} cells (6) (7)vs CD24^{lo} cells (31), and it is likely that differences in antibodies and staining protocol account for these apparent discrepancies.

Stingl et al also report that culturing primary MECs leads to 100% Sca1 positivity, which has not been observed in our studies (7). Stingl et al use a longer digestion process (8 h vs. 1 h) with different enzymatic solutions including cholera toxin, which may select for a somewhat different population of cells and impact the outgrowth numbers. To rule out the possibility that culturing the cells confounds the results, we analyzed freshly isolated primary MECs after *in vivo* irradiation and demonstrated that 4 Gy increases the Sca1^{+} population. These data suggest that while differences in isolation protocols may have significant impact on absolute marker percentages, this may have less impact on relative difference between samples.

Building on our experience with putative progenitors using established protocols, we used both the SP phenotype and Sca-1 as surrogate progenitor cell markers, and the standard daily dose of radiation that would be delivered during a course of radiation therapy for breast cancer, i.e. 2 Gy, and found that the percentages of both SP and Sca1^{+} cells increased in primary Balb/c MECs. At higher doses of radiation, the percentage of progenitor cells declined suggesting that 6 Gy is sufficient to kill both progenitor and non-progenitor cells. Our findings demonstrate that almost exclusively, Sca1^{+} cells give rise to colonies in Matrigel, and clinically relevant doses of radiation failed to reduce the number of colonies formed by Sca1^{+} cells. Of interest is the observation that in preliminary experiments using MECs from either mammary tumors from p53-null mice

or mammary hyperplasias from Wnt-1 transgenic mice, $Sca1^-$ cells were capable of limited colony formation which is not diminished by 2-4 Gy of radiation. (Mei Zhang and Jeffrey M. Rosen unpublished data).

These data are consistent with observations regarding stem cell irradiation in the small intestine summarized eloquently by Dr. Christopher Potten (4). Previous findings from our laboratory and others suggested that while the side population and $Sca1^+$ population may *contain* lineage ancestor stem cells, most SP and $Sca1^+$ populations represent progenitors (5, 10, 30): i.e. potential stem cells or transiently amplified cells that may be more resistant to radiation than the lineage ancestor stem cells. Similar to the findings in the intestine, the single cell stem cell phenotype in the normal mouse mammary gland, $CD24^+CD29^+$, is sensitive to clinically relevant doses of radiation. Interestingly, a similar population in human breast tumor MCF-7 cells is enriched after irradiation highlighting a potential difference between normal and tumor stem cells. Further studies are required to determine if this subpopulation of MCF-7 cells will exhibit increased tumorigencity in xenografts.

β-Catenin has been implicated as a stem cell survival factor in several systems including neural crest cells, gastrointestinal crypts, epidermal follicles, and hematopoetic stem cells (32-36). Inhibition of ß-catenin signaling in mammary alveolar progenitors leads to the inhibition of mammary development and pregnancy-induced proliferation implicating ß-catenin as a stem cell survival factor in the mammary gland (37). In addition, it has been shown that the SP-enriched fraction is increased in the mammary gland of MMTV- Wnt-1 and MMTV-ΔNß-catenin transgenic mice, and that ectopic Wnt ligands increase the SP fraction in MECs after 3 days in culture (24). A recent study

reported the initial development of a small molecule Wnt/ß-cat pathway inhibitor, that downregulates ß-catenin/TCF mediated gene expression through its interaction with the cyclic-AMP responsive element binding protein (38). This molecule, ICG-001, has been shown to inhibit ß-catenin/TCF mediated transcription of survivin, which has been shown to be upregulated in many cancers. Survivin is regulated by APC in intestinal crypts, where it may limit the stem cell population on the basis of its localization and regulation (39). We showed that survivin is selectively upregulated by radiation in $Sca1^{+}$ cells. Since apoptosis is a minor component of radiation induced cell death in solid tumors at low doses (22), this finding potentially suggests a non-apoptosis related role for survivin in these cells. Indeed, such a role has recently been described in colon cancer cell lines where survivin was shown to assist cancer cells in escaping replicative senescence by enhancing telomerase activity (40).

In conclusion, this is the first study to demonstrate radioresistance of progenitor cells in the mouse mammary gland. On the basis of our findings and of the studies presented here, we suggest that the Wnt/ß-catenin signaling pathway may be an attractive target for directed anti-stem cell therapeutics. Although ß-catenin is not commonly mutated in human breast cancers, several studies have implicated components of the Wnt signaling pathway in human breast cancer pathogenesis and prognosis (41-44), and Annayan et al have recently reported a direct link between Wnt-1 signaling and the DNA damage response primary human epithelial cells (45) These studies underscore the potential for treatment strategies that target pathways such as Wnt/ß-catenin that are responsible for self-renewal (46).

MATERIALS AND METHODS

Cell Culture. All animals used were used in accordance with an IRB approved protocol and euthanized prior to gland collection. MECs were isolated from 6-8-week-old wild-type Balb/c and from C57BL/6 mice (Harlan, Indianapolis, IN) containing a floxed exon III ß-catenin allele (Catnb $^{loxP(ex3)}$, Dr. Makoto Taketo, Kyoto University, Kyoto, Japan). The MECs generated stabilized ß-catenin on excision in culture with an adenovirus-driven Cre recombinase, AdCre1 (47), at a multiplicity of infection of 50, as determined by an adenovirus-expressing *Escherichia coli* ß-galactosidase ([Adß-gal]; AdCre1 and Adß-gal (M. Abdelative and M. Schneider, Baylor College of Medicine, Houston, TX). In addition, MECs were isolated from transgenic Wnt-1 mice with mammary hyperplasias (Dr. Yi Li, Baylor College of Medicine, Houston, TX) and from wild-type MECs from mice of the same genetic background as the Wnt-1 transgenic mice. All 10 glands were isolated. For primary MEC culture (Figures 1a, 1c, 2 and 3), The epithelial cell fraction was isolated as described previously (48). Primary MECs were plated at a density of 2.5×10^5 cells/cm^2 in 6-well plates that had been coated with 100 µL/cm^2 of serum/fetuin (20% fetal calf serum [FCS], Summit Biotechnology, Fort Collins, CO) and 1 mg/mL of fetuin (Sigma, St. Louis, MO). Cells were left for 2 days in F12 plating medium (5 µg/mL of insulin, 2 µg/mL of hydrocortisone, 5 ng/mL of epidermal growth factor, 50 µg/mL of gentamycin, 100 U penicillin/streptomycin, and 10%fetal calf serum) (GIBCO-BRL, Grand Islands, NY). After 48 h in the plating medium, MECs were maintained in stem cell-promoting neurobasal medium (GIBCO-BRL) containing 4 µg/mL of B-27 supplement (GIBCO-BRL), 20 ng/mL of basic fibroblast growth factor (Invitrogen), 20 ng/mL of insulin-like growth factor-I

(Invitrogen), and 20 ng/mL of epidermal growth factor (Invitrogen). Cells were irradiated on day 4. Primary MECs were irradiated in 60-mm culture dishes using a ^{137}cesium cell irradiator at doses of 2, 4, 6, or 8 Gy. Control cells were sham irradiated. On day 5, cells were trypsinized (10X, JRH Biosciences) for 3 min; washed in Hanks' balanced salt solution (GIBCO-BRL), 2% FBS, and 10 mM HEPES ($HBSS^{+}$); and stained with Hoechst-33342 (Sigma) at a final concentration of 5-15 μg/mL for 60 min (8) before being analyzed by flow cytometry.

Fresh digestion was performed using the following protocol: in vivo irradiation (Sham or 4 Gy, Figure 1d and e) was performed 48 h prior to isolation of MECs. After administration of 0.2ml 1.2% Avertin, mice were immobilized in the supine position to allow uniform dosimetry to the glands using a small animal ^{137}Cesium irradiator irradiator. Glands were isolated from 7-10 week old Balb/c female mice and minced into small pieces. The minced tissue was digested with 2mg/ml collagenase (Roche) and 300U/ml hyuronidase (Sigma) in HBSS+ buffer, using 1g tissue/10ml digestion solution at 37C, 100 rpm for 2 h. The tissue slurry was further digested in 0.25% trypsin-EGTA for 1 min, 5 mg/ml dispase (Roche Diagnostics) for 5 min. The suspension was washed and pelleted twice at 800rpm (103rcf, or 14.4g). The cell pellet was collected for antibody staining with 2 ul each of the Biotin-Conjugated Mouse Lineage Panel (BD Pharmingen) containing Mouse CD3e, CD11b, CD45R, Ly-6G and Ly-6C, and TER-199, was used to stain 10^6 cells in 10 ul on ice for 15 min. Streptavidin conjugated PE-Cy5.5 (Caltag laboratories) were used at 1:200 dilution on ice for 15 min. CD29 (BD Pharmingen) was used at 1:200 at room temperature for 60 min. A goat anti-rat Alexa 488 secondary antibody (Molecular Probes) was used at 1:200 dilution at room

temperature for 30 min. CD24-PE (BD Pharmingen) was used on ice for 15 min at 1:200. MCF-7 cells were radiated on plastic 48 h prior to analysis and examined for SP or marker expression by flow cytometry.

Immunofluorescence. Primary Balb/c MECs were cultured, treated, and stained with PE-conjugate Sca1 antibody (BD Pharmingen, San Diego, CA) diluted at 1:200. $Sca1^+$ and $Sca1^-$ were sorted directly onto glass slides (FisherBiotech ProbeOn Plus, Hampton, NH) at 500 cells each. The sorted cells were fixed in 4% paraformaldehyde for 15 min and stained with phospho-H2AX antibody (1:200) and a secondary Texas Red-conjugated anti-rabbit antibody (Molecular Probes, Carlsbad, CA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (Vector, Burlingame, CA), and coverslips were mounted with a SlowFade light antifade kit (Molecular Probes). Images were captured at 100x using a Zeiss CCD camera.

Flow Cytometry. Samples were prepared for flow cytometry using antibodies or Hoescht-33342, resuspended in $HBSS^+$, and filtered through a 0.45-µm cell filter into polypropylene tubes (Fisher) containing 0.5 µg/mL of propidium iodide (Sigma) to exclude dead cells. Analysis and sorting were performed on a triple laser MoFlo (Cytomation, Fort Collins, CO). The Hoechst dye was excited at 350 nm, and its fluorescence measured at 450 nm/20BP filter blue and 675 nm EFLP optical filter red, as described previously (8). Activated ß-catenin was measured using the anti-non-phospho-ß-catenin antibody, clone 8E4 (Upstate Cell Signaling Solutions, Charlottesville, VA). Data analysis was performed with FlowJo software, version 4 (Tree Star, Inc, Ashland, OR.).

Survivin Polymerase Chain Reaction Methods. The survivin primer sequences were 5′-AAGAACTACCGCATCGCCACC for survivin and 5′-AGCCAGCTCCGCCATT for survivin rev. Cells were harvested 24 h after irradiation. SYBR green quantitative PCR was performed using the Applied Biosystems ABI 7500 real-time PCR system.

Statistical Analysis. Statistical comparisons were performed using the students' T-test in excel. All p-values are 2-sided.

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FIGURE LEGENDS

Figure 1

Clinically relevant doses of radiation increased the percentage of progenitor cells (%SP and Sca1$^+$) in primary MEC culture and human MCF-7 cells. (A) MECs were isolated from Balb/c mice, cultured for 3 days, irradiated, and analyzed for %SP by Hoechst 33342 staining and flow cytometry. Radiation selectively increased the progenitor fraction (%SP) (p = 0.015 [2 Gy], 0.008 [4 Gy], and 0.05 [6 Gy], by the two-tailed t-test). (B) MCF-7 cells were analyzed for %SP by Hoechst 33342 staining and flow cytometry. Radiation selectively increased the progenitor fraction (%SP) (p = 0.05 0 vs 4 Gy by the two-tailed t-test). (C) Cells were analyzed for Sca1 in the SP 24 h after irradiation. Radiation selectively increased the Sca1$^+$ (progenitor) fraction within the SP by killing the more sensitive Sca1$^-$ (nonprogenitor) cells ($p < 0.05$; Sca1$^+$ to Sca1$^-$ at 0 vs. 2–8 Gy). The differences in effects of doses of 2 Gy vs. higher doses were not significant. (D) Anesthetized Balb/c mice were immobilized supine and mammary glands (entire ventral surface) were irradiated. MECs were isolated 48 h after irradiation and analyzed immediately for Sca1 by flow cytometry. Radiation selectively increased the Sca1$^+$ (progenitor) fraction and decreased the Sca1$^-$ (nonprogenitor) cells. *p < 0.0001.

Figure 2

In vivo radiation increased the percentage of CD24$^+$CD29$^+$ positive cells from MCF-7 cells but not uncultured MECs

(A) Freshly digested MECs were analyzed for lin$^-$ CD24$^+$CD29$^+$ 48 h after in vivo irradiation. The CD24$^+$CD29$^+$ population is sensitive to radiation. (F) MCF-7 cells were

irradiated and analyzed for lin$^-$ CD24$^+$CD29$^+$ by flow cytometry. Radiation selectively decreased the lin-CD24$^+$CD29^{lo} fraction cells (p = 0.003 0 Gy vs. 2 Gy and p = 0.0002 0 Gy vs 4 Gy.

Figure 3.

Radiation induced more DNA damage foci in Sca1$^-$ cells 2 h after irradiation. Sca1$^+$ and Sca1$^-$ cells from Balb/c MECs were sorted onto glass slides after irradiation with 2 Gy and immunostained with anti-phospho-H2AX (scale bar, 10 μm). There were significantly more DNA-damaged foci in the Sca1$^-$ population than in the Sca1$^+$ population (3.7-fold difference, $^{**}p < 0.05$).

Figure 4.

Clinically relevant doses of radiation led to an increased percentage of SP cells in primary mouse MECs isolated from mice with a gain-of-function, conditionally stabilized ß-catenin allele and from Wnt1 transgenic mice than in control cells. (A) MECs from Wnt-1 transgenic mice at 16 weeks of age and wild-type mice of the same background were stained with Hoechst 33342, and the %SP was analyzed using flow cytometry as described. $^*p = 0.08$ for 0 Gy Wnt vs. 2 Gy Wnt, two-tailed *t*-test. MECs from mice treated with AdCre recombinase to generate stabilized ß-catenin or an AdLacZ control vector were stained with Hoechst 33342, and the %SP was analyzed using flow cytometry as described. $^*p = 0.001$ for 0 Gy wild type (WT) vs. Wnt and $p = 0.04$ for 2 Gy WT vs. Wnt. **Radiation selectively activated ß-catenin and survivin in Sca1$^+$ cells**. (B) Quantitative assessment of activated ß-catenin signaling was assessed by flow

cytometry after staining for Sca1 and unphosphorylated ß-catenin. Real-time PCR for survivin expression was performed 24 h after irradiation in $Sca1^{+}$ and $Sca1^{-}$ cells.

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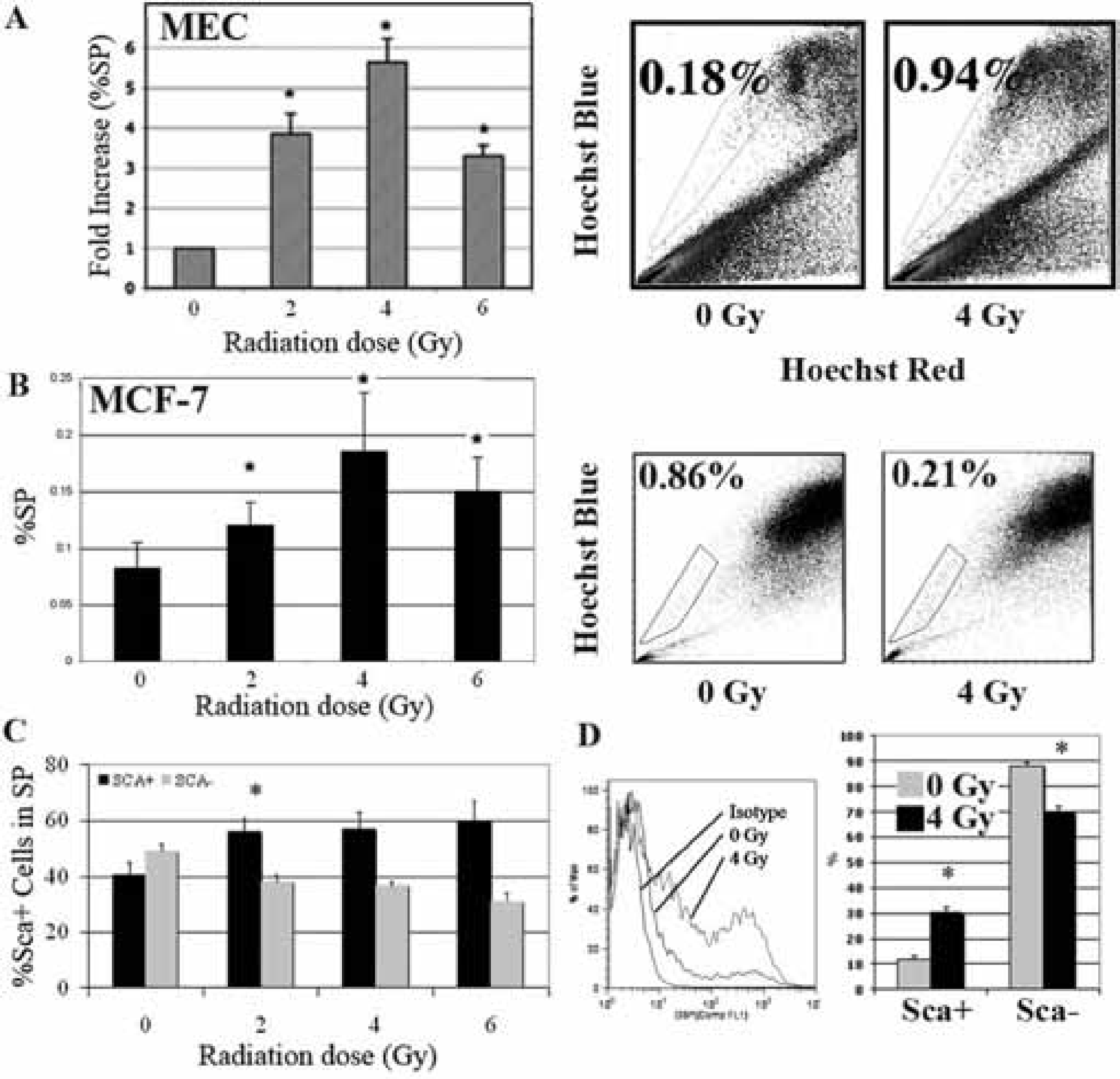
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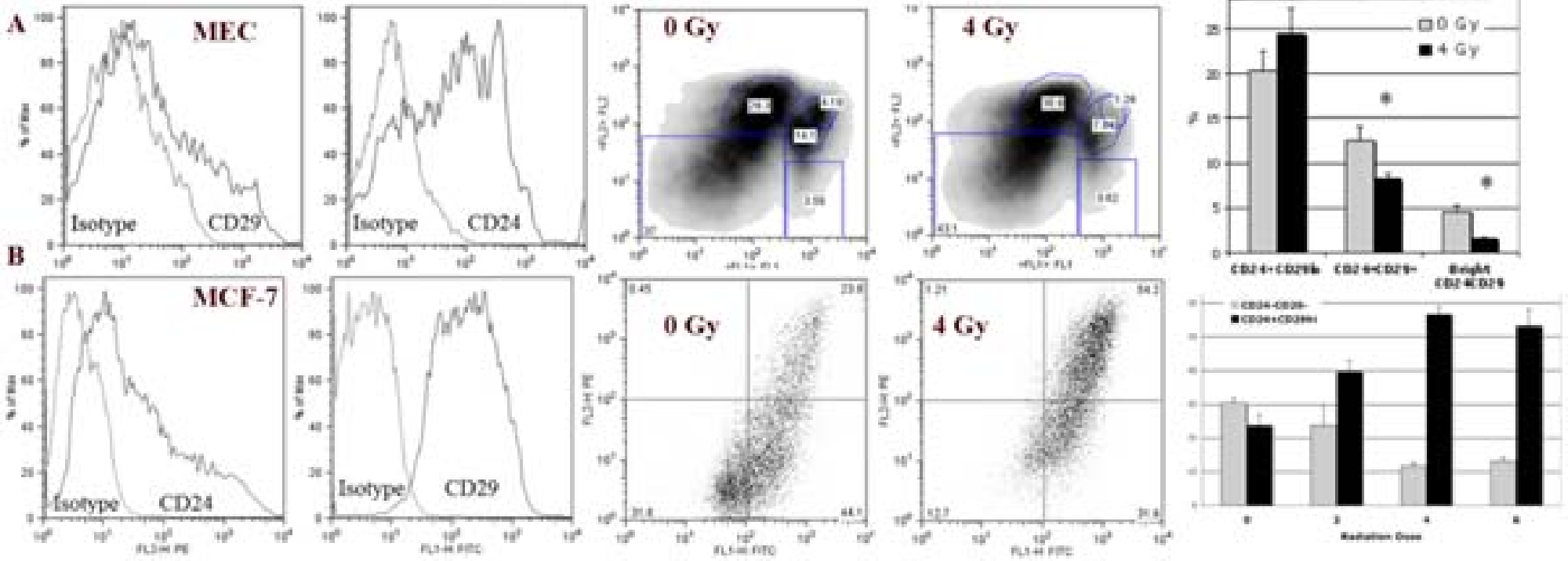
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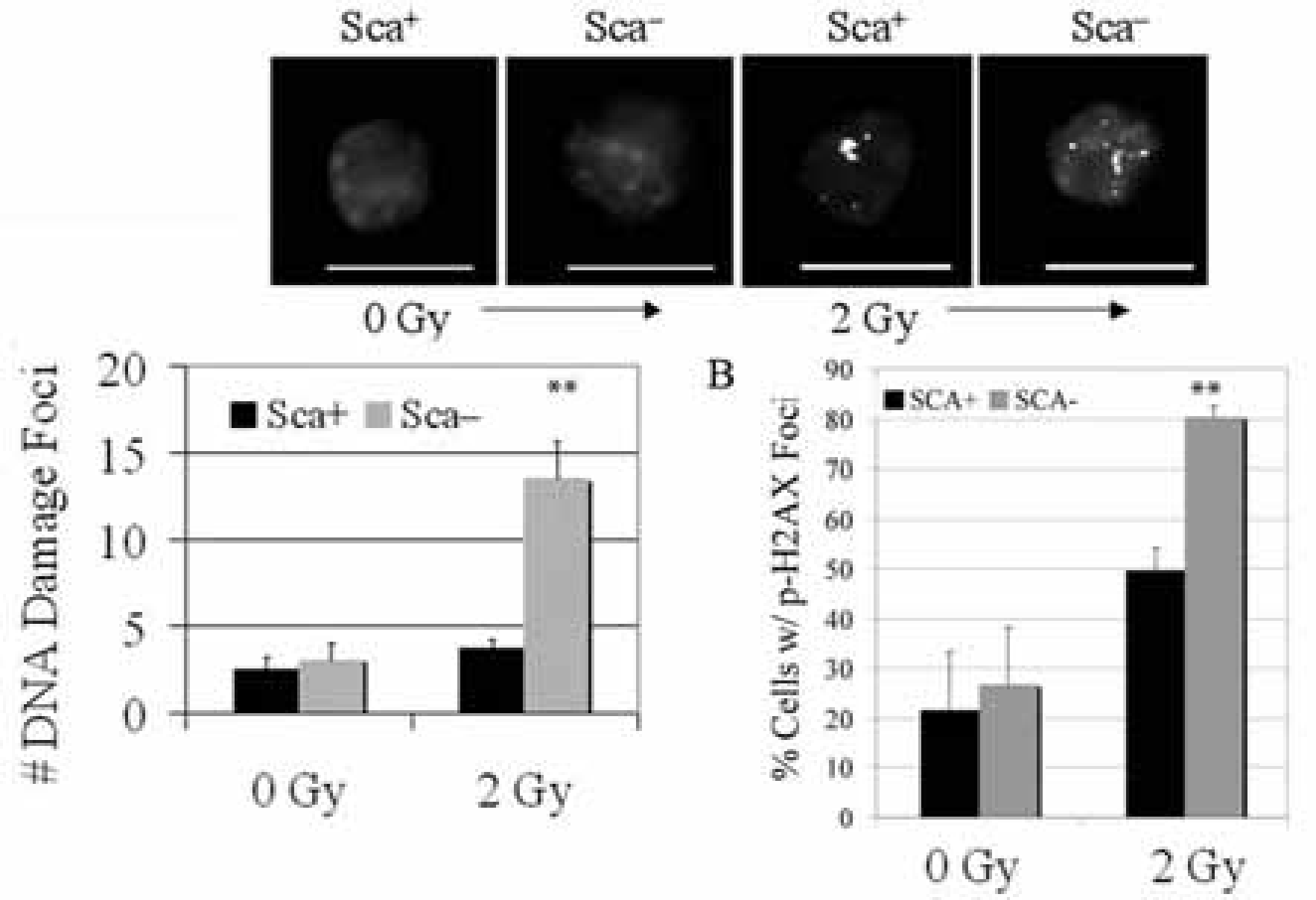
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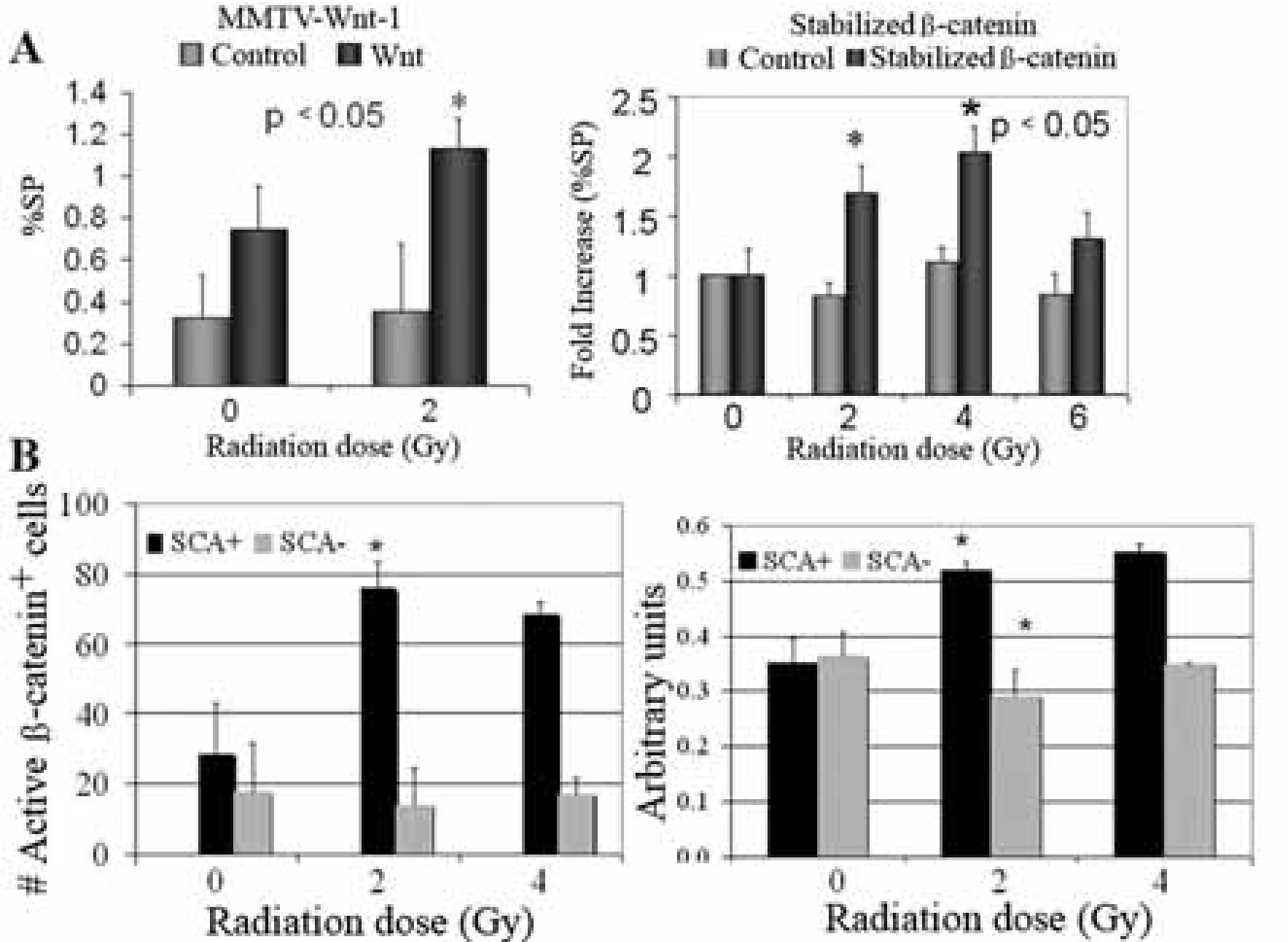
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Wnt/ß-Catenin Mediates Radiation Resistance of Stem Cell Antigen-1 Positive Progenitors in an Immortalized Mammary Gland Cell Line

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Nonstandard abbreviations used: CD, COMMA-D; CDßgeo, COMMA-D ß-geo; $Sca1^{+}$, stem cell antigen-1 positive; $Sca1^{-}$, stem cell antigen-1 negative; LRCs, label-retaining cells; SP, side population; K6, keratin 6; K18, keratin 18; MSCV, murine stem cell virus; GFP, green fluorescent protein; ß-GAL, ß-galactosidase; ß-CAT, ß-catenin; ß-ENG, ß-engrailed; Gy, Gray (1 Gy = 100 rads).

Abstract

Progenitor cells are thought to be targets of tumorigenesis and may acquire self-renewal properties due to activation of signaling pathways, such as the Wnt/ß-catenin pathway, known to be critical for stem cell self-renewal. We hypothesized that progenitor cells may be resistant to conventional cancer therapy, such as radiation, and therefore, may be responsible for recurrent disease. Using the putative progenitor cell marker Sca1, we were able to isolate a discrete subpopulation of $Sca1^+$ multipotent progenitor cells from the immortalized COMMA-D ß-geo murine mammary cell line. Expression of active ß-catenin enhanced self-renew preferentially in the $Sca1^+$ cells, while suppressing ß-catenin with a dominant negative, ß-engrailed, decreased self-renewal of the $Sca1^+$ cells. At a clinically relevant dose, the $Sca1^+$ cells were resistant to radiation (2 Gy). $Sca1^+$ cells contained fewer gamma-H2AX-positive DNA damage foci following radiation, displayed higher levels of endogenous ß-catenin, and selectively upregulated survivin after radiation. Taken together, these data underscore the importance of the Wnt/ß-catenin pathway in regulating progenitor cell survival, and self-renewal, and the radiation damage response, and in addition, suggest that the COMMAD ß-geo cell line may provide a useful model to study the signaling pathways that control mammary progenitor cell regulation.

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Introduction

Breast epithelium is capable of completely and functionally regenerating upon transplantation. This impressive renewal capacity has been ascribed to the function of a multipotent mammary gland stem/progenitor cell population that resides and persists throughout the mammary parenchyma. Elucidating the role of stem/progenitor cells in preneoplasia may be crucial understanding the etiology of breast cancer, and may lead to better chemopreventative strategies. Mutations or epigenetic changes either in long-term stem cells or their immediate progeny, the transit amplifying multipotent progenitors, have been suggested to be the foundation of malignancy (Clarke and Fuller, 2006). However, functional studies of progenitor cells in a preneoplastic model have not been explored.

In normal development, the Wnt pathway has been shown to be important in stem cell survival and maintenance in early embryogenesis (Wang and Wynshaw-Boris, 2004); in maintaining the stem cell pool in the adult skin (Silva-Vargas et al., 2005), intestinal epithelium (Pinto and Clevers, 2005b; Pinto et al., 2003); as well as regulating hematopoietic stem cells in their niche environment (Rattis et al., 2004). Dysregulation of the Wnt/ß-catenin pathways in stem cell regulation has been proposed to be one of the signaling pathways responsible for carcinogenesis in the hematopoietic system, the intestine and the epidermis (Reya and Clevers, 2005). Studies in chronic myelogenous leukemia indicate that the elevated levels of nuclear ß-catenin exist in a minor population of progenitor cells, resulting in their enhanced capacity for self-renewal and increased leukemic potential (Jamieson et al., 2004). Further evidence of dysregulation of stem/progenitor cell self-renewal and maintenance by the Wnt/ß-catenin pathway have been demonstrated in lung cancer, colorectal cancer, and gastrointestinal cancer

(Brabletz et al., 2005; He et al., 2005; Mishra et al., 2005; Reya and Clevers, 2005; Yardy and Brewster, 2005).

Stem cell antigen-1 (Sca1), a marker of hematopoietic stem cells, is one of the putative markers used to isolate and enrich for mammary gland progenitors. Previous studies by our laboratory have demonstrated that primary mammary gland progenitor cells isolated by using Sca1 lack differentiation markers, are enriched in label-retaining, relatively quiescent cells, and have enhanced outgrowth activity compared to the Sca1 negative cells (Welm et al., 2003).

The COMMA-D β-geo (CDßgeo) cell line is a clonal, immortalized, cell line isolated from the parent COMMA-D (CD), preneoplastic, mouse mammary cell line by both transfection and selection using a dominant-selectable gene transfer vector and by limiting dilution (Deugnier et al., 2006). Recent studies by Deugnier et al have demonstrated that the CD ßgeo cells contain a permanent population of mouse mammary epithelial progenitor cells with basal characteristics, which express putative stem cell markers such as stem cell antigen 1 (Sca1), CD24 and CD49f, among others. These investigators demonstrated that Sca1 positive cells have at least 4-fold enrichment in outgrowth potential than Sca1 negative cells. CD ßgeo cells at passage 18 were reported to generate “normal appearing mammary outgrowth upon in vivo transplantation”. In addition, these investigators have observed that Sca1 positive cells were able to form 3D acini on Matrigel, whereas Sca1 negative cells grew poorly. Staining for cell type specific markers indicated that the 3D-acini contained K5+ basal cells, K8+ luminal as well alpha-SMA+ myoepithelial cells within the acinus (Deugnier et al., 2006). The parental CD cell line was originally derived from primary mammary epithelial cells of mid-pregnant Balb/c mice and was shown previously by transplantion into the cleared fat pads of syngeneic mice to resulted in outgrowths containing all of the epithelial cell types that make up a mammary gland (Danielson

et al., 1984). Subsequent clonal derivatives, such as the HC11 cell line and others have been extensively used to study lactogenesis and hormonal regulation in mammary epithelial cells. The CD cell line has been shown to contain mutations in both alleles of p53 (Jerry et al., 1994).

Herein we report that CD β-geo cells contain a minor population of Sca1 positive cells that are able to self-renew, and differentiate asymmetrically. In addition, we report that $Sca1^{+}$ cells sustain less DNA damage as indicated by the formation of gamma-H2AX foci, contain higher levels of non-phosphorylated or active ß-catenin, and are more resistant to radiation than Sca1- cells. To date, this is the first report where functional studies of progenitor cells have been carried out in a preneoplastic model to explore mechanisms of therapeutic resistance in the mammary gland.

Results

COMMA-D ß-geo Sca1$^+$ cells are capable of self-renewal and expansion

The CDßgeo cell line, originally isolated from the midpregnant mammary gland of Balb/c mice, has been shown to retain stem and multipotent progenitor cell characteristics (Danielson et al., 1984; Deugnier et al., 2006). Because CDßgeo cells retain stem/progenitor cell properties in vivo, we sought to distinguish self-renewing cells from cells lacking this capacity. The CDßgeo cells are heterogeneous, containing Sca1$^+$, and Sca1$^-$ populations. Routinely 13-20% Sca1$^+$ cells were observed in culture (Fig. 1a). Clonogenic assays were used to determine the replicative competence of CDßgeo subpopulations. Sca1$^+$ cells form 47-fold more colonies than Sca1$^-$ cells ($p < 0.00002$, Fig. 1b). Consistent with these data, Deugnier et al also have shown that the Sca1- cells are unable to form attachments in Matrigel, and give rise to an organized spheroid structure (Deugnier et al., 2006).

The hallmark of stem and multipotent progenitor cells is the ability to asymmetrically self-renew thereby maintaining the progenitor pool and giving rise to differentiated daughter cells. To examine whether CDßgeo cells were able to divide asymmetrically, cells were first sorted into Sca1$^+$ and Sca1$^-$ subpopulations. After culturing for 96 hours, both of the subpopulations are able to proliferate (Figure 4d). The original Sca1$^+$ cells were able to give rise to both Sca1$^+$ (75% ± 4.2) and Sca1$^-$ cells (24% ± 4.1) (Fig. 1c). In contrast, the Sca1$^-$ cells were less bipotent, giving rise to predominantly Sca1$^-$ cells (94% ± 1.5) (Fig. 1d).

CDßgeo cells can self-renew in suspension culture

To further explore the self-renewing potential of CDßgeo progenitor cells, we used the mammosphere in vitro self-renewal assay described by Dontu et al. (Dontu et al., 2003), which is

based upon the hypothesis that progenitor cells are able to survive in anchorage-independent conditions. Ten thousand cells were plated on low adherence plates in serum free media supplemented with EGF and bFGF as growth stimulants. Approximately 0.1-0.6% of the CDßgeo cells were able to form mammospheres. By retroviral tagging experiments, Dontu et al. have previously shown that mammospheres are clonally derived, and not a result of aggregation (Dontu et al., 2003). In addition, mammospheres are now known to comprise a heterogeneous population of cells, with multipotent mammary stem cells within the core, and surrounded by progenitor cells in various stages of differentiation. Therefore, we examined the expression patterns of putative stem cell markers in the secondary mammospheres, such as CD49f, TIE2, Keratin 6 (K6), and epithelial specific markers Keratin 14 (K14) and Keratin 18 (K18). In previous studies, CD49f has been associated with multi-potent mammary gland progenitors (Stingl et al., 2005), TIE2 has been shown to identify quiescent hematopoietic stem cells and is thought to function by maintaining these cells in the bone marrow niche (Arai et al., 2004). K6 is expressed within the body cells of the developing mammary terminal end buds, and overexpressed in Wnt-1 murine mammary tumors together with Sca1 (Grimm et al., 2006; Li et al., 2003). K14 is an epithelial-specific marker used to identify basal epithelial cells while K18 identifies luminal epithelial cells. In the secondary mammospheres examined, CD49f was expressed mainly in the center of the mammosphere (Fig. 2b). K14 staining was also localized in the center of the mammosphere (Fig. 2c). Both TIE2 and K6 were randomly distributed throughout the mammospheres (Fig. 2d, e) and luminal K18 positive cells were not detected (data not shown).

ß-catenin enhances self-renewal

Studies using transgenic mice expressing Wnt1 or stabilized ß-catenin, such as the MMTV-Wnt1 and MMTV-DN89b-catenin, have indicated that hyperplasias and tumors rapidly develop and that they are enriched in cells expressing stem and/or progenitor markers (Imbert et al., 2001; Li et al., 2003). Additional studies from our laboratory using a dominant negative chimera, ß-engrailed, which specifically suppressed ß-catenin signaling without affecting its cell-cell adhesion function, inhibited cell survival in lobuloalveolar progenitors (Tepera et al., 2003). These studies suggested that ß-catenin plays a critical role in stem or multipotent progenitor cell self renewal.

To determine if there is any difference in self-renewing efficiency between $Sca1^+$ and $Sca1^-$ subpopulations, and to examine whether ß-catenin is required for self-renewal, we first transduced the CDßgeo cells with control (MSCV-IRES-GFP), stabilized ß-catenin (MSCV-ß-catenin-IRES-GFP), or the dominant-negative chimera, ß-engrailed (MSCV-ß-engrailed-IRES-GFP), and then sorted into $Sca1^+$ and $Sca1^-$ populations. The efficiency of secondary mammosphere formation (the number of mammospheres per the number of seeded cells) generated by both the $Sca1^+$ and $Sca1^-$ populations was quantitated as described in Materials and Methods. The control $Sca1^+$ population demonstrated a significant increase in the efficiency for mammosphere formation compared to the control $Sca1^-$ population (GFP control $Sca1^+$ vs. $Sca1^-$ 1.9 -fold, $*p < 0.02$, Fig. 3). In addition, transduction with stabilized ß-catenin enhanced $Sca1^+$ mammosphere formation compared to the GFP control (GFP $Sca1^+$ vs. ß-catenin $Sca1^+$, 1.6- fold increase, $**p < 0.008$, Fig. 3), while ß-engrailed decreased the number of mammospheres in both the $Sca1^+$ and the $Sca1^-$ populations (5.4-fold decrease, $p < 0.004$, Fig. 3). These data indicate that stabilized ß-catenin selectively enhances the mammosphere-forming capacity in the $Sca1^+$ cells while the dominant-negative ß-engrailed depletes the mammosphere-forming capacity.

Sca1$^+$ cells are resistant to clinically relevant doses of radiation

We and others have hypothesized that cancer progenitor cells that remain after therapy may lead to recurrence. To determine whether Sca1$^+$ progenitor cells may be resistant to clinically relevant doses of radiation, we examined the replicative competence of CDßgeo progenitors after radiation and then compared the clonogenic potential of Sca1$^+$ and Sca1$^-$ cells. Clonogenic assays are classically used to demonstrate radioresistance, since cells that can form colonies after radiation are clearly still competent to reproduce and, therefore, represent a fraction of cells that persist and may lead to tumor recurrence (Pawlik and Keyomarsi, 2004). Sca1$^+$ and Sca1$^-$ CDßgeo cells were sorted into 96 well plates containing growth factor reduced – Matrigel at 500 cells per well. Plating efficiency (number of colonies/number of cells plated) for colony formation from Sca1$^-$ cells was 0% while plating efficiency for colony formation from Sca1$^+$ cells was 2-4% at 500-cell density (Fig. 4a), therefore, Sca1$^-$ cells were unable to form colonies. The Sca1$^+$ surviving fraction after 2 Gy was 100%, although the colony size after 2 Gy is reduced by 33% ($p < 0.0001$, Fig. 4a).

It is thought that progenitor cells may be resistant to conventional cancer therapy, such as radiation in part, due to their quiescence. To test our hypothesis that progenitor cells may be resistant to radiation, we used a number of approaches. Cell cycle analysis of Sca1$^+$ and Sca1$^-$ subpopulations was performed using 7-AAD and pyronin Y, which stain DNA and RNA, respectively, to discriminate G0 from different stages within G1 as shown in previous studies (Schmid et al., 2000; Xin et al., 2005). Although the presence of a minor quiescent subpopulation within either sample cannot be excluded by this method, there were no significant differences in the cell cycle profiles between Sca1$^+$ and Sca1$^-$ cells 24 hours following radiation. Both

populations were actively cycling, and occupied G0, G1, and S/G2/M similarly (Fig. 4b). Cellular proliferation was compared between the $Sca1^+$ and $Sca1^-$ cell populations using a MTT cell proliferation assay. $Sca1^+$ cells demonstrate increased proliferation after radiation over time in culture (*p value < 0.0001, Fig. 4c). The rate of apoptosis was similar between the two populations by using annexin V staining. There was also no significant difference in senescence between the $Sca1^+$ and $Sca1^-$ cells (data not shown).

In addition, we examined the proliferation rates of $Sca1^+$ and $Sca1^-$ cells by generating growth curves over a period of six days. The CDßgeo cells were radiated at 0 Gy (sham radiation) and 4 Gy, then separated into $Sca1^+$ and $Sca1^-$ populations by FACS sorting. The cells were then plated at sub-confluent density, and cell number was determined every 48 hours. The $Sca1^-$ cells had decreased proliferation compared to the $Sca1^+$ cells (86% of 0 Gy $Sca1^+$). Following radiation at 4 Gy, the $Sca1^+$ cells showed a decrease in proliferation rate from 48 to 96 hours, but their proliferation was nearly identical to the control 0 Gy $Sca1^+$ cells by 144 hours (96% of 0 Gy $Sca1^+$). In contrast, $Sca1^-$ cells exhibited a much lower proliferation rate at 4 Gy through 144 hours (12% of $Sca1^+$). Following radiation at 4 Gy, the $Sca1^+$ cells exhibited a lag in proliferation as compared to the 0 Gy control $Sca1^+$ cells, but recovered and matched the control $Sca1^+$ cells at 144 hours. In contrast, following radiation, the $Sca1^-$ cells not only exhibited decreased proliferation following radiation, but by 144 hours had not recovered as compared to the 0 Gy control (Fig. 4d).

To determine whether there are differences in radiation-induced DNA damage between the $Sca1^+$ and $Sca1^-$ cells, we examined DNA-damage foci using gamma-H2AX as a DNA-damage marker. Immunostaining of gamma-H2AX immediately following radiation at 4 Gy showed that the $Sca1^-$ cells contained more gamma-H2AX foci as compared to the $Sca1^+$ cells.

Quantification of gamma-H2AX foci confirmed that Sca1- cells contained 60% more foci than the $Sca1^+$ cells following radiation (Fig. 5).

Alteration in ß-catenin localization and upregulation of survivin expression following radiation

To determine the impact of the Wnt/ß-catenin signaling pathway on radioresistance, we examined whether there is any intrinsic difference between the $Sca1^+$ and $Sca1^-$ cells. First we compared the level of ß-catenin in these two subpopulations. Using an antibody specific for the non-phosphorylated (or active) form of ß-catenin, we compared the level of ß-catenin using flow cytometry analysis (Fig. 6a). There is a significantly lower percentage of cells with detectable levels of non-phosphorylated ß-catenin in the $Sca1^-$ cells (~1.2%) as compared to the $Sca1^+$ cells (~95%) (Fig. 6a). Deconvolution microscopy revealed that non-phosphorylated ß-catenin is mainly localized around the cell membrane in the $Sca1^+$ cells, whereas it is difficult to detect ß-catenin in the $Sca1^-$ cells. Following radiation at 4 Gy, the non-phosphorylated ß-catenin is found mainly in and around the nucleus rather than around the cell membrane. It appears that $Sca1^+$ and $Sca1^-$ cells are intrinsically different with respect to the level of non-phosphorylated ß-catenin. Interestingly, the localization of ß-catenin was altered dramatically following radiation in the $Sca1^+$ cells (Fig. 6b).

Survivin, a bifunctional member of the inhibitor of apoptosis family, has been shown to be a direct target of TCF/ß-catenin signaling. In addition, studies in pancreatic cancer, brain tumors, as well as rectal cancer (Kami et al., 2004; Rodel et al., 2005; Zhen et al., 2005), indicate that survivin may play a role in radioresistance. To determine whether survivin is differentially regulated in response to radiation, we examined survivin expression in both $Sca1^+$ and $Sca1^-$ cells using real time PCR. CDßgeo cells were transduced with control (ß-galactosidase), ß-

catenin, and ß-engrailed, respectively; followed by FACS sorting into $Sca1^+$ and $Sca1^-$ populations. Radiation selectively enhanced survivin expression in $Sca1^+$ cells at 2 Gy (ß-gal control $Sca1^+$, 0 Gy vs. 2 Gy, 1.5 fold, *p value < 0.02, Fig. 6c). Since it is known that ß-catenin directly activates survivin, transduction with stabilized ß-catenin significantly increased survivin expression (2 fold, $p < 0.03$, Figure 6c). Survivin level increased further following radiation in the ß-catenin transduced cells (ß-cat $Sca1^+$, 0Gy vs. 2 Gy, **p value < 0.04, Fig. 6c). Interestingly, transduction with ß-engrailed, the dominant-negative variant, did not decrease the basal level of survivin. However, radiation did not elevate survivin level in the ß-engrailed transduced cells.

Discussion

Deciphering the biological and molecular events in mammary preneoplasia will provide potential means to block progression to malignancy. To date, studies using several immortalized cancer cell lines, including the human breast cell line MCF-7, have indicated that they contain a subpopulation of highly tumorigenic cells, which retain stem/progenitor-like properties (Kondo et al., 2004; Patrawala et al., 2005; Ponti et al., 2005). However, functional studies of progenitor cells in a preneoplastic model have not been explored.

It is somewhat surprising to find a subpopulation of progenitor cells that are $Sca1^{+}$ within an immortalized mammary cell line. Similar to the findings from Deugnier et al. (Deugnier et al., 2006), we observed a consistent subpopulation (13-20%) of $Sca1^{+}$ cells within the CDßgeo cell line, which is comparable to previous data from our laboratory demonstrating a 15-20% $Sca1^{+}$ subpopulation in primary mammary epithelial cells. Not only is the $Sca1^{+}$ subpopulation from the primary mammary epithelial cells enriched in long term label-retaining cells and side population cells which efflux the Hoechst dye, they are also enriched in transplant potential compared to the $Sca1^{-}$ cells, suggesting that Sca1 is a marker of progenitor cells (Welm et al., 2003). In contrast, a recent report by Shackleton et al. has demonstrated that while they are able to isolate primitive mammary stem cells using CD49f/CD29, cells expressing high Sca1 are not enriched in CD49f/CD29 population. In addition, they have found that the $Sca1^{hi}$ cells did not enrich for outgrowth frequency, while CD49f/CD29 cells with increased outgrowth potential were $Sca1^{lo}$ and not $Sca1^{-}$. However, in our previous transplantation studies (Welm et al., 2002) the entire $Sca1^{+}$ population was separated from the $Sca1^{-}$ population by magnetic bead separation or using a $Sca1^{-}$ EGFP knockout mouse and sorting for EGFP positive and negative cells. Thus, these two results are actually consistent and suggest that the $Sca1^{+}$ population is heterogeneous

and contains within it a subpopulation with increased outgrowth potential. A comparison between the percent Sca1 found in our laboratory and these recent studies (Shackleton et al., 2006; Stingl et al., 2006) indicates that the percent Sca1 reported by Shackleton et al. is 3-fold higher than reported in our previous studies. It is likely that different cell preparation and FACS gating protocols may account for such a large discrepancy. These discrepant results may in fact represent differences in nomenclature highlighting the importance of careful discussion and presentation of flow output and gates. Interestingly, Shackleton et al. report that Sca1 is not enriched in the CD49f/CD29 population. This again suggests that within the mammary gland, there could be a number of subpopulations of stem and/or progenitor cells, each with overlapping or distinct characteristics. Whereas CD49f/CD29 may represent a more primitive stem cell population, the Sca1 subpopulation may encompass a more committed progenitor population (Woodward et al., 2005). In both cases, the majority of these cells are in S/G2/M and only a minority of these populations contain label retention cells or LRCs, thus, additional markers will be required to identify the quiescent stem cell population. Furthermore, while these antigens are putative progenitor cell markers, there are no functional data suggesting that either CD49f or CD29 is necessary for stem cell fate. In fact, the CD49f mammary-specific knockout displayed no overt phenotype, while the CD29 conditional knockout exhibited only a limited lactation defect (Klinowska et al., 2001; Li et al., 2005).

The mammosphere assay (free floating spherical aggregates with the potential to self-renew and to differentiate into all cell types of the mammary gland), analogous to neurospheres (Reynolds and Weiss, 1996), is an assay for self-renewal, based on the idea that stem cells may survive in anchorage- independent conditions while differentiated cells need attachment to survive, and die by anoikis when they lose contact with extracellular matrix. Dontu et al. (Dontu

et al., 2003) reported that when grown in conditions similar to neurospheres, four in 1000 cells isolated from a normal reduction mammoplasty survive in anchorage-independent conditions, and were able to form mammospheres with only bFGF and EGF as growth stimuli. In addition, secondary and tertiary mammospheres have been shown to be more homogeneous and are further enriched in self-renewal capacity (Dontu et al., 2003). We have observed that the CDßgeo cells display a similar ratio: approximately 1 out of 1000 CDßgeo cells gives rise to a mammosphere. Consistent with the idea that multipotent stem cells are within the core of the mammosphere, we have observed that CD49f is found in cells at the center. However, TIE2 and K6 expression appeared to be random, suggesting that perhaps these are not markers exclusively for putative stem cells, are associated with differentiated cells, and perhaps are staining for cells at various stages of differentiation. We have not observed significant mammosphere enrichment following secondary or tertiary passaging. Moreover, when we compared the self-renewal between the $Sca1^{+}$ and $Sca1^{-}$ subpopulations, the $Sca1^{+}$ cells showed increased mammosphere-forming efficiency. While overexpression of ß-catenin increased the mammosphere-forming efficiency further, depleting endogenous ß-catenin by using the dominant negative chimera, ß-engrailed, decreased mammosphere-forming efficiency dramatically, suggesting that ß-catenin is required in the self-renewal process. This observation is consistent with a number of reports indicating that ß-catenin is important in regulating stem/progenitor cell maintenance in the intestinal epithelium, in neurogenesis, in the cilliary margin of the eye, to list just a few examples (Inoue et al., 2005; Lie et al., 2005; Pinto and Clevers, 2005a). Activation of the Wnt/ß-catenin pathway in Wnt-1 transgenic mice induces an enrichment of side population progenitor cells both in vitro and in vivo (Liu et al., 2004). In addition, caveolin- deficient mice have been

reported to display an increase in progenitor cells, due to an indirect activation of the Wnt/ß-catenin pathway (Sotgia et al., 2005).

It is widely accepted that radiation-induced cell death typically occurs via mitotic catastrophe during cell division rather than apoptosis. However, increasing evidence suggests that stem/progenitor cells evade cell death by a number of mechanisms, such as quiescence and drug-efflux conferred by expression of the ABC-family of membrane transporters (Dean et al., 2005). The cell cycle profiles of the CDßgeo $Sca1^+$ and $Sca1^-$ cells suggest that the $Sca1^+$ cells are not quiescent stem cells, but rather, are actively proliferating transit amplifying progenitor cells. In addition, the increased clonogenicity and proliferation potential of the $Sca1^+$ cells suggest that these cells have enhanced ability to sustain radiation in contrast to the $Sca1^-$ cells. Furthermore, preliminary studies indicate that radiation does not deplete the efficiency of mammosphere formation, suggesting that radiation at 2 Gy does not affect the self-renewal capacity of $Sca1^+$ CDßgeo cells at clinically relevant doses (S. L. Poplack and J.M. Rosen, unpublished data).

The differences in the levels of DNA damage as well as differences in proliferation following radiation between the $Sca1^+$ and $Sca1^-$ cells may be due to in part to their intrinsic chromatin structure as well as the differences in the endogenous levels and the localization of ß-catenin, which has been suggested to play a role in proliferation as well as DNA damage response. Studies in human breast cancer have shown that Wnt signaling triggers DNA damage response, followed by events that result in conversion of primary human mammary epithelial cells (Ayyanan et al., 2006). In addition, studies in colorectal cancer have shown that PARP1, a DNA binding protein that plays a role in DNA repair, recombination and proliferation, binds to TCF/ß-catenin, and its expression tightly correlates with overexpression of ß-catenin, c-myc, and

cyclin D1 in colorectal cancers (Nosho et al., 2006). Moreover, recent studies have suggested that ß-catenin plays a critical role in histone modification and subsequent activation of proliferation associated genes by binding to TRRAP/TIP60 and SET1 type chromatin modifying enzymes (Sierra et al., 2006). Taken together, these studies suggest that ß-catenin plays a key role in regulating DNA damage repair and proliferation preferentially in the $Sca1^+$ cells. Conversely $Sca1^-$ cells, which express a markedly decreased level of active ß-catenin, do not show the same level of proliferation following radiation.

The increased radioresistance observed in the CDßgeo $Sca1^+$ cells may be conferred also in part, by elevated levels of survivin, a direct target of ß-catenin/TCF signaling. Survivin was shown to be strongly expressed in human and mouse embryonic intestinal crypts, whereas its expression is absent in TCF-4 knockout animals (Kim et al., 2003). Expression of non-destructible ß-catenin mutant increased survivin expression and protected colorectal cancer cells against UV-induced apoptosis, suggesting that TCF/ß-catenin mediated expression of survivin impresses a stem cell-like phenotype to colonic crypt epithelium coupling enhanced cell proliferation with resistance to apoptosis. The selective increase in survivin expression level in $Sca1^+$ cells expressing ß-catenin suggests that survivin may be a mechanism regulated in part by the Wnt/ß-catenin pathway, which increases radioresistance in the progenitor cells. Moreover, since apoptosis is a minor component of radiation-induced cell death in solid tumors at low doses (Hall, 2000). This finding potentially suggests a non-apoptosis related role for survivin in these cells. Indeed, such a role has recently been described in colon cancer cell lines where survivin was shown to assist cancer cells in escaping replicative senescence by enhancing telomerase activity (Endoh et al., 2005). However, the lack of suppression in ß-eng transduced cells suggests that survivin may be regulated in part by other pathways. Notably, the stress response NF-

kappaB pathway has been shown to regulate survivin expression. Kawakami et al. (Kawakami et al., 2005) have shown that inhibiting the NF-kappaB pathway resulted in suppression of survivin expression and caused apoptosis of Tax-expressing malignant CTLL-2 T-cells. Furthermore, recent studies by Vong et al. (Vong et al., 2005) provided evidence for the role of survivin in regulating mitosis via a differential ubiquitination mechanism.

In conclusion, it has been speculated that progenitor cells have evolved the ability to resist conventional breast cancer therapy including radiation therapy, and resistant cells that remain after multimodality therapy may result in breast cancer recurrence. This study demonstrates that the CDßgeo cell line maintains a population of self-renewing, asymmetrically dividing, progenitor cells. We were able to use the CDßgeo cells as a model to establish that the Wnt/ß-catenin pathway impacts self-renewal and proliferation of progenitor cells. Finally, we provided evidence for radioresistance of progenitor cells, and more importantly, that the Wnt/ß-catenin pathway enhances radioresistance. The data presented suggest that progenitor cell response to radiation can be specifically targeted. Immortalized cell lines such as CDßgeo that contain a subpopulation of multipotent progenitors may be useful preclinical models to identify targets for which a therapeutic window could be examined and novel targeted clinical therapies designed.

Materials and Methods:

Antibodies

Anti-CD49f rat monoclonal antibody was obtained from BD Pharmingen. Anti-TIE2 polyclonal antibody was obtained from Santa Cruz Biotechnologies. Anti-K6 polyclonal antibody was obtained from Covance. Anti-K8 rat monoclonal antibody was obtained from Developmental Studies Hybridoma Bank, Univ. Iowa. Anti-non-phosphorylated ß-catenin antibody, clone 8E4, and gamma-H2ax antibody were obtained from Upstate Cell Signaling Solutions, Charlottesville, VA.

Immunofluorescence

Samples on glass slides: Samples were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 minutes, permeabilized with 5% TritonX-100 for 20 minutes, followed by a 1-hour blocking step in 20% goat serum/ 3% bovine serum albumin / 0.1% Tween-20. Paraffin embedded sections: 3 μm sections were deparaffinized in xylene, then rehydrated through a graded ethanol series. Immunostaining was performed after microwave antigen retrieval (20 minutes) in 10 mM sodium citrate and blocking in 20% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween-20. Primary antibody staining was performed either for 1 hour at room temperature or overnight at 4°C. Immunofluorescence staining was detected with the appropriate secondary antibodies conjugated with Texas Red, Alexa 568, or Alexa 488 (Molecular Probes, Eugene, OR, USA), performed at room temperature for 45 minutes. Nuclei were counter-stained with DAPI (Vector, Berlingame, CA) and coverslips were mounted with SlowFade Light Antifade Kit (Molecular Probes). Images were captured at 100X using a Zeiss CCD camara or captured by deconvolution microscopy using a Zeiss AxioVert S100 TV

microscope and a DeltaVision restoration microscopy system (Applied Precision, Inc.). For high-resolution deconvolved images, captured raw images were deconvolved with the DeltaVision constrained iterative algorithm. All images were digitally processed for presentation with Adobe Photoshop.

Cell Line

COMMA-D ß-geo cell line passages 21-27 was kindly provided by Dr. D. Medina at Baylor College of Medicine. The cells are grown in DMEM/F12 (Invitrogen) pH 7.6, with 2% Adult Bovine Serum (Gemini Bioproducts, CA), 2.383 mg/ml HEPES (Invitrogen), 5 μg/ml Gentamycin (Sigma), 10 μg/ml Insulin (Invitrogen), and 5 ng/ml EGF (Invitrogen).

Retroviral Transduction

Stabilized ß-catenin (Montross et al., 2000) and ß-engrailed constructs were cloned into the pS2 retroviral backbone (kindly provided by Aguilar-Cordova, Baylor College of Medicine, Faustinella et al., 1994) as described in Tepera et al. (Tepera et al., 2003). Stabilized ß-catenin and ß-engrailed were also cloned into the pMSCV-IRES-GFP retroviral backbone (kindly provided by Dr. Greg Hannon, Cold Spring Harbor, NY. Transduction procedures was performed as described in Tepera et al. (Tepera et al., 2003). Briefly, 293T packaging cells (ATCC) were transiently transfected with target constructs and pCL-Eco (Imgenex Corp) using Fugene (Roche) according to the manufacturer's guidelines. Forty-eight hours after transfection (day 3), medium was collected from transfected 293T cells, filtered through 0.22 mm syringe filter, and applied to CDßgeo cells in a 1:1 ratio (1 plate 293T to 1 plate CDßgeo). The cells were spun at 3,000 g in a Marathon 6K clinical centrifuge (Fisher Scientific) on a swinging platform

rotor for 30 minutes. The retroviral supernatant was removed from CDßgeo cells and replaced with media.

Flow Cytometry

Samples were prepared for flow cytometry using anti-Sca1 antibody conjugated to FITC or PE (BD Pharmingen). Cells are incubated with Sca1 antibody for 15 minutes on ice, resuspended in HBSS+ (HBSS (Invitrogen), 2% fetal bovine serum (JRH Biosciences) and 100 mM HEPES (Invitrogen)), and filtered through a 45 μm cell filter into polypropylene tubes containing 0.5 μg/ml propidium iodide (Sigma) to exclude dead cells. Analysis and sorting were performed on a triple laser MoFlo (Cytomation, Fort Collins, CO). Data analysis was performed on FlowJo version 4, Tree Star, Inc.

Flow cytometry analysis for non-phosphorylated ß-catenin:

Cells were sorted directly onto glass slides (FisherBiotech ProbeOn Plus, Hampton, NH) at 500 cells each for immunofluorescence staining. For non-phosphorylated ß-catenin flow cytometry analysis, cells were directly sorted into 5ml polypropylene tubes (Falcon), and then fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA). Primary antibody was used at 1:200 dilution in 1X Cytoperm at 4°C overnight, and Alexa 488-conjugated secondary antibody was used at 1:500 at room temperature for 30 minutes. Cells were washed and resuspended in 1X Cytoperm, and strained through a 45-μm filter before flow cytometry analysis.

Cell Cycle Analysis

Cell cycle analysis was performed according to the protocol described in (Xin et al., 2005). Briefly, cells were resuspended in 0.5 ml of NASS buffer (0.15 M NaCl 5 mM sodium EDTA 0.5% BSA fraction V 0.1 M phosphate citrate buffer, pH 4.8) containing 0.02% saponin and 10 g/ml 7-aminoactinomycin D at room temperature for 20 minutes. Cells were washed with PBS and resupended in NASS containing 0.02% saponin and 10 μg/ml actinomycin D at 4°C for 5 minutes. Pyronin Y (0.5 μl of 1 g/ml) diluted in distilled water was added, and samples were incubated at 4°C for 10 minutes before flow cytometry analysis.

Mammosphere assays

Mammosphere growth conditions were based on Dontu et al. (Dontu et al., 2003). Briefly, 10,000-20,000 CDßgeo cells/well were grown in 6-well Ultra Low Attachment plates (Corning, NY) in serum free DMEM/F12 (Invitrogen) supplemented with 20 ng/ml bFGF (Invitrogen), 20 ng/ml EGF (Invitrogen), and B27 (Invitrogen). The cells were fed every 3-4 days, and passaged using 0.05% trypsin, 0.53 mM EDTA-4Na (Invitrogen). Following trypsin digestion, single cells from the primary mammospheres were plated at 1000 cells/well. After passage 2, the mammospheres were counted using a Leica dissecting scope. Two researchers counted colonies independently and the numbers were averaged. In preparation for immunofluorescence staining, mammospheres were cyto-spun onto glass coverslips, fixed for 15 minutes on ice in 4% paraformaldehyde, and stored in 70% ethanol. Otherwise, paraffin embedded sections of mammospheres were sectioned at a thickness of 3 μm.

Clonogenic assays

For clonogenic assays using sorted $Sca1^+$ or $Sca1^-$ cells, CDßgeo cells were sham irradiated or

treated with 2 Gy approximately one hour prior to trypsinization. CDßgeo cells were trypsinized for 3 minutes, washed in HBSS+ and stained for 30 minutes with FITC conjugated anti-Sca1-antibody (BD Pharmingen). Cells were sorted into 96 well plates (round-bottom, Costar) containing 10 µl of growth factor reduced Matrigel (Discovery Labware) per well. Plates were prepared with Matrigel while on ice and kept on ice prior to sorting. After sorting, 50-100 µl of CDßgeo media was carefully added to each well. After 7-11 days, plates were fixed in glacial acetic acid and methanol (10 minutes, 1:2) and stained with crystal violet. Fixing and staining were performed with great care in order not to dislodge the Matrigel from the well. Crystal violet was rinsed by adding water to wells overnight rather than repeat washings to reduce perturbation of wells. Two researchers counted colonies independently and numbers were averaged.

MTT assay

CDßgeo cells were sorted by flow cytometry into $Sca1^{-}$ and $Sca1^{+}$ subpopulations as described previously. After sorting the cells were radiated at the indicated radiation intensity, 0, 2, 4, and 6 Gy followed by plating in 6 well plates at a density of 2500 cells per well in triplicates. Cells were serum-starved (0.1% adult bovine serum), and stimulated to proliferate in serum (5% adult bovine serum). The MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, (Sigma Aldrich ^{R}) assay was performed at 24, 48 and 144 hours after serum starvation.

Growth Curve

The relative proliferation between the CDßgeo $Sca1^{+}$ and $Sca1^{-}$ cells was examined by generating a growth curve. The CDßgeo cells were radiated at 0 (sham) and 2 Gy followed by

separation of the cells into $Sca1^+$ and $Sca1^-$ sub-populations by FACS sorting directly into 6-well plates at 50,000 cells per well. The cells were replenished with fresh media containing 5% adult bovine serum every 48 hours. The cells were harvested by trypsinization every 48 hours, and the cell number was determined using a hemocytometer.

Real Time PCR

Survivin primer sequences: survivin for 5'aagaactaccgcatcgccacc and survivin rev 5' agccagctccgccatt. Cells were harvested 24 hours following radiation. SYBR green quantitative PCR was performed using Applied Biosystems ABI 7500 Real Time PCR system.

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Figure Legends

Fig. 1

CDßgeo $Sca1^+$ cells are capable of self-renewal and expansion. (a) CDßgeo cells were stained with FITC-conjugated antibody against Sca1, followed by analysis by flow cytometry. Two populations can be distinguished based on Sca1 fluorescence: $Sca1^+$ 13.1 %; and $Sca1^-$, 67.4% (b) Cells are sorted into $Sca1^+$ and $Sca1^-$ populations directly into 96 well plates containing growth factor reduced – Matrigel at a 500-cell density, and clones were counted after 10 days. The $Sca1^+$ cells gave rise to 47± 14 colonies in Matrigel, while the $Sca1^-$ produced no colonies. Colonies were stained with crystal violet and counted by two independent researchers. (d) CDßgeo $Sca1^+$ population give rise to 75% ± 4.2 $Sca1^+$ and 24% ± 4.1 $Sca1^-$ cells. (d) CDßgeo $Sca1^-$ cells remain mostly $Sca1^-$ after culture, gave rise to 94% ± 1.5 $Sca1^-$ cells and very few, 6% ± 1.45 $Sca1^+$ cells.

Fig. 2

CDßgeo cells can self-renew in suspension culture and express putative stem cell marker. (a) Bright field. ~1 in 600-1000 CDßgeo cells form a mammosphere. Scale bar = 100 μm (b) CDßgeo mammospheres are cyto-spun onto glass slides, and immunostained for CD49f (red). Nuclei are visualized with DAPI (blue). Scale bar = 20 μm (c) CDßgeo mammospheres are immunostained for TIE-2. Nuclei are visualized with DAPI (blue). Scale bar = 20 μm (d) CDßgeo mammospheres express K14 (red). Nuclei are visualized with DAPI (blue). Scale bar = 50 μm. (E) CDßgeo mammospheres express Keratin 6 (green). Nuclei are visualized with DAPI (blue). Scale bar = 50 μm

Fig. 3

Stabilized ß-catenin expression enriches for stem/progenitor cells by increasing the number of mammospheres as well as promoting mammary outgrowths from transplanted CDßgeo cells. CDßgeo cells transduced with GFP (control), ß-catenin, or ß-engrailed were FACS sorted into $Sca1^+$ and $Sca1^-$ populations and grown in suspension at a density of 20,000 cells per well for 14 days. The mammospheres were passaged once after 7 days, and the passage 2 mammospheres were counted using a Leica dissecting scope. GFP control $Sca1^+$ vs. $Sca1^-$ 1.9 -fold, $*p < 0.02$; GFP $Sca1^+$ vs. ß-catenin $Sca1^+$, 1.6- fold increase, $**p < 0.008$. The efficiency of mammosphere formation is calculated as the number of mammospheres per the number of seeded cells. Data was collected from three individual experiments performed in triplicate sets.

Fig. 4

Radiation does not alter colony formation from $Sca1^+$ cells in Matrigel. (a) 500 $Sca1^-$ or $Sca1^+$ cells treated with either 0 or 2 Gy were sorted using flow cytometry into 96 well plates containing 10 µl of Matrigel. Representative images from wells containing 500 cells are shown. Scale bar = 2 mm. $Sca1^+$ 0 Gy vs. $Sca1^+$ 2 Gy, $p < 0.07$ by two-tailed t-Test. (b) CDßgeo cells were irradiated at either 0 or 2Gy, sorted into $Sca1^+$ and $Sca1^-$ populations. Sorted cells were stained with 7-AAD and pyronin Y to discriminate G0 from different stages within G1, see methods. There is no obvious difference in the cell cycle profiles between the $Sca1^+$ and $Sca1^-$ population, at either 0 or 2 Gy. Cell cycle was analyzed using flow cytometry. Statistics were assessed on FlowJo version 4, Tree Star, Inc. (c) Significant differences in proliferation 144 hours following radiation. MTT assay was performed on the sorted $Sca1^+$ and $Sca1^-$ cells after radiation at 144 hours at 0 (sham), 2, 4, and 6 Gy. Following radiation, the sorted cells were

serum starved (0.1% adult bovine serum) for 48 hours, and then stimulated to proliferate with serum (5% adult bovine serum). See Methods for details. The bars indicate average OD (absorbance) and the error bars are the standard deviation of three replicate OD measurements within each group. The star indicates significant differences in growth rate comparing $Sca1^{+}$ and $Sca1^{-}$ cells radiated at equivalent doses. There were statistically significant differences in growth rate between the $Sca1^{+}$ and $Sca1^{-}$ cells at 0 Gy, 2 Gy, and 4 Gy, *p value < 0.0001, two-tailed t-Test. (d) Growth properties of $Sca1^{+}$ and $Sca1^{-}$ cells at 0 and 4 Gy. Cells were directly sorted into 6-well plates at 50,000 cells per well, and replaced with fresh media with serum (5% adult bovine serum) every 48 hours. Determination of cell number was performed every 48 hours using a hemocytometer. The graph shows the growth curve of 0 Gy $Sca1^{+}$ (blue), 0 Gy $Sca1^{-}$ (red), 4 Gy $Sca1^{+}$ (yellow), 4 Gy $Sca1^{-}$ (green). Each data point represents the mean ± SEM of 3 experiments in triplicate. The different cell culture conditions may account for the differences in the kinetics of recovery.

Figure 5. Radiation induces more DNA damage foci in $Sca1^{-}$ cells. (a) $Sca1^{+}$ and $Sca1^{-}$ cells were sorted directly onto glass slides following radiation at 2 Gy, immunostained with anti-gamma-H2AX (red). Nuclei are stained with DAPI (blue). Scale bar = 5 μm. (b) There were significantly more DNA-damage foci in the Sca- population (4 Gy $Sca1^{+}$ vs. $Sca1^{-}$, 2.8-fold difference, $p < 0.0001$). A minimum of 200 - 300 cells were counted in each group.

Fig. 6

Differences in ß-catenin level and localization between $Sca1^{+}$ and $Sca1^{-}$ subpopulations. (a) $Sca1^{+}$ and $Sca1^{-}$ subpopulations were stained with non-phosphorylated ß-catenin and analyzed

by flow cytometry with an Alexa-488 antibody against ß-catenin. ~95% of the $Sca1^+$ cells contain non-phosphorylated ß-catenin, while only ~1.2% of the Sca1- cells contain non-phosphorylated ß-catenin. (b) Immunostaining for non-phosphorylated ß-catenin in $Sca1^+$ and $Sca1^-$ cells at 0 Gy (sham irradiation) and 4 Gy. ß-catenin is visualized in green, and the nuclei are stained with DAPI in blue. Scale bar = 5 μm. Images were captured by deconvolution microscopy using a Zeiss AxioVert S100 TV microscope and a DeltaVision restoration microscopy system (Applied Precision, Inc.). For high-resolution deconvolved images, captured raw images were deconvolved with the DeltaVision constrained iterative algorithm.
(c) ß-Catenin regulates survivin expression following radiation. CDßgeo cells were tranduced with control vector, ß-catenin, or ß-engrailed, and then irradiated at 0 or 2 Gy. Cells were harvested after 24 hours, FACS sorted into $Sca1^+$ and $Sca1^-$ subpopulations, and RT-PCR was performed using the ABI real time PCR system. ß-gal control $Sca1^+$, 0 Gy vs. 2 Gy, 1.5 fold, *p value < 0.02; ß-cat $Sca1^+$, 0Gy vs. 2 Gy, **p value < 0.04. Data was obtained from 5-7 individual experiments performed in triplicate sets.

Figure 1.

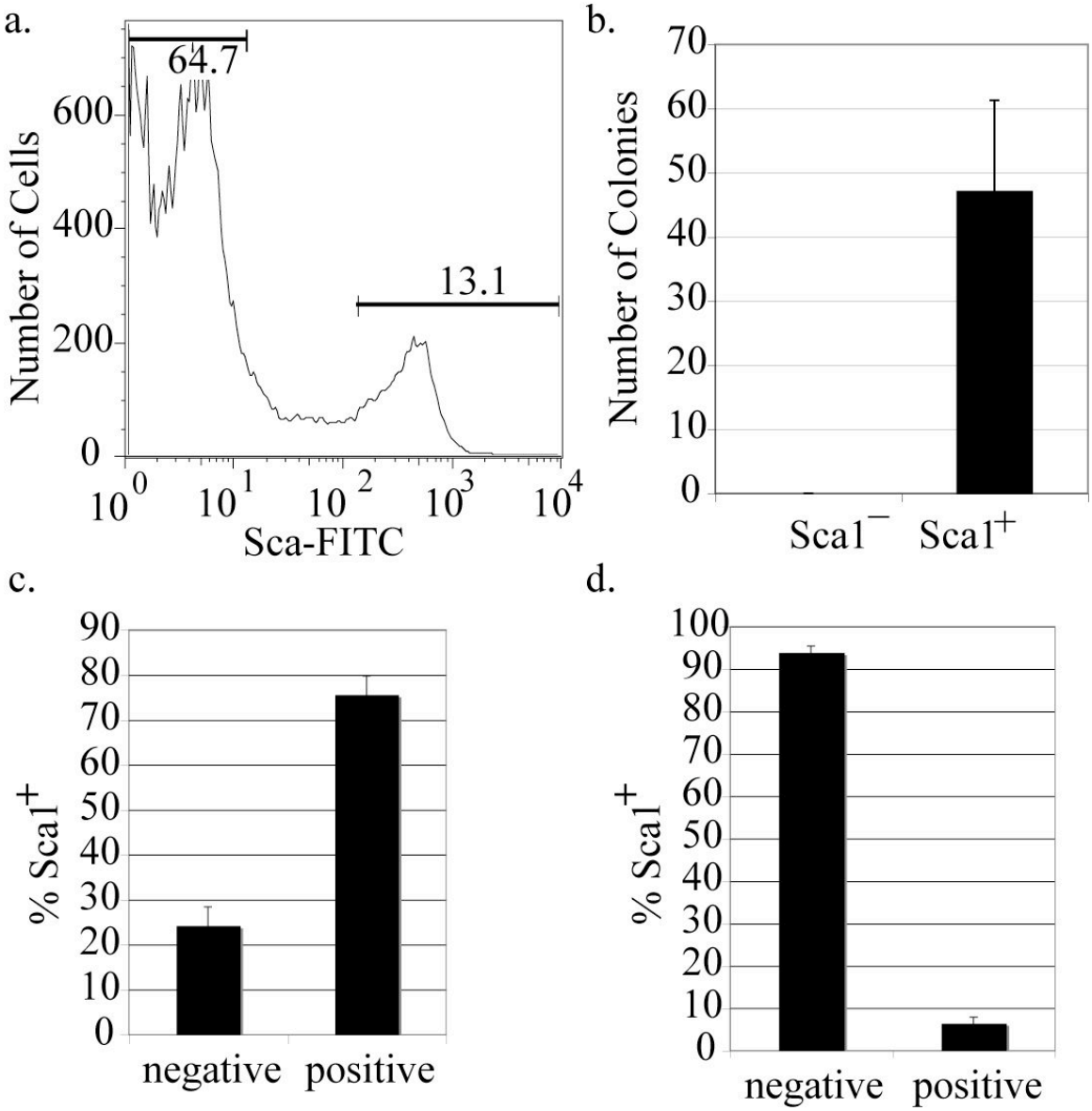

a.
64.7
13.1
Number of Cells
600
400
200
0
10^0 10^1 10^2 10^3 10^4
Sca-FITC
b.
Number of Colonies
70
60
50
40
30
20
10
0
Sca1$^-$ Sca1$^+$
c.
% Sca1$^+$
90
80
70
60
50
40
30
20
10
0
negative positive
d.
% Sca1$^+$
100
90
80
70
60
50
40
30
20
10
0
negative positive

Figure 2.

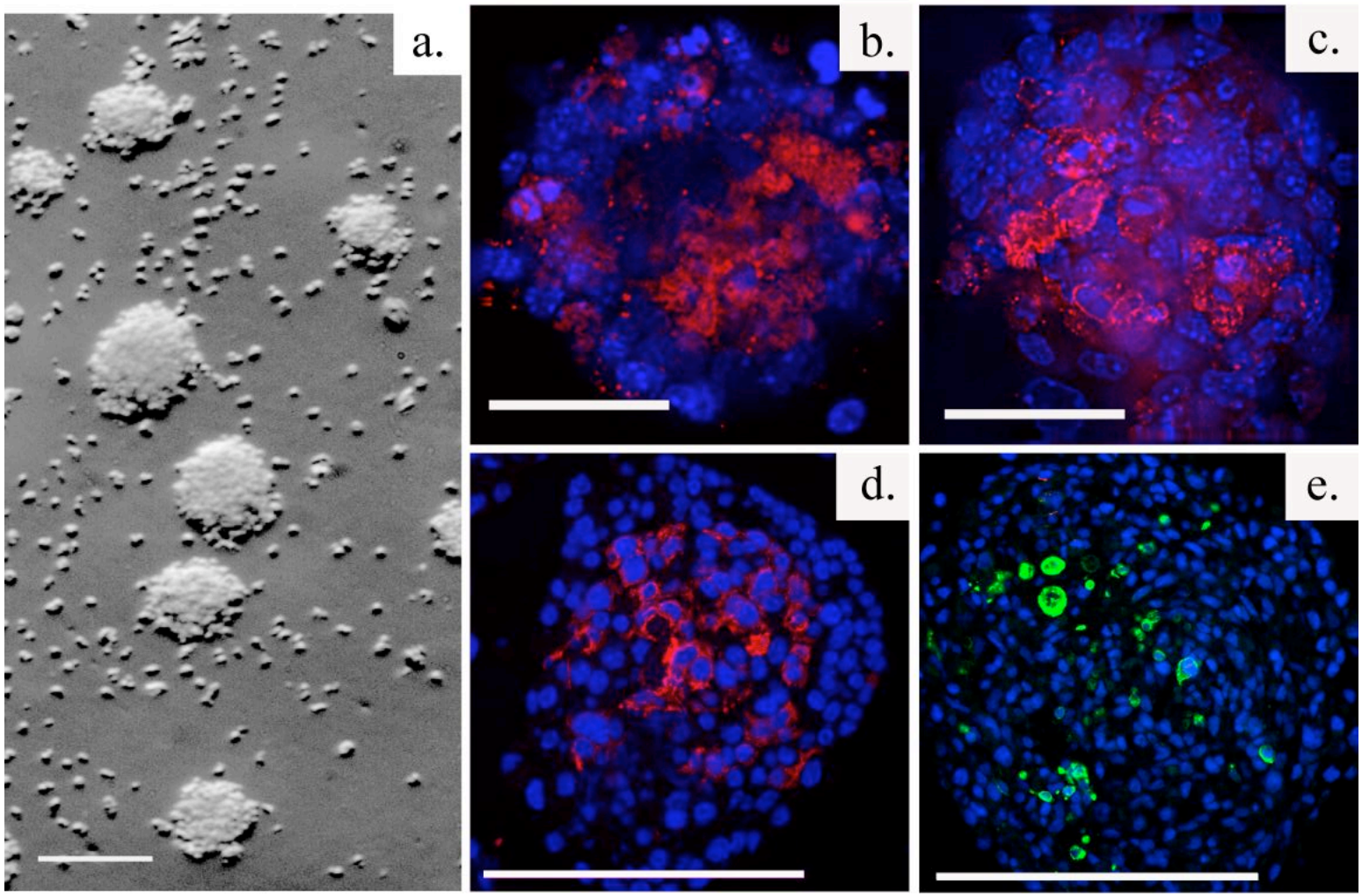


Figure 3.

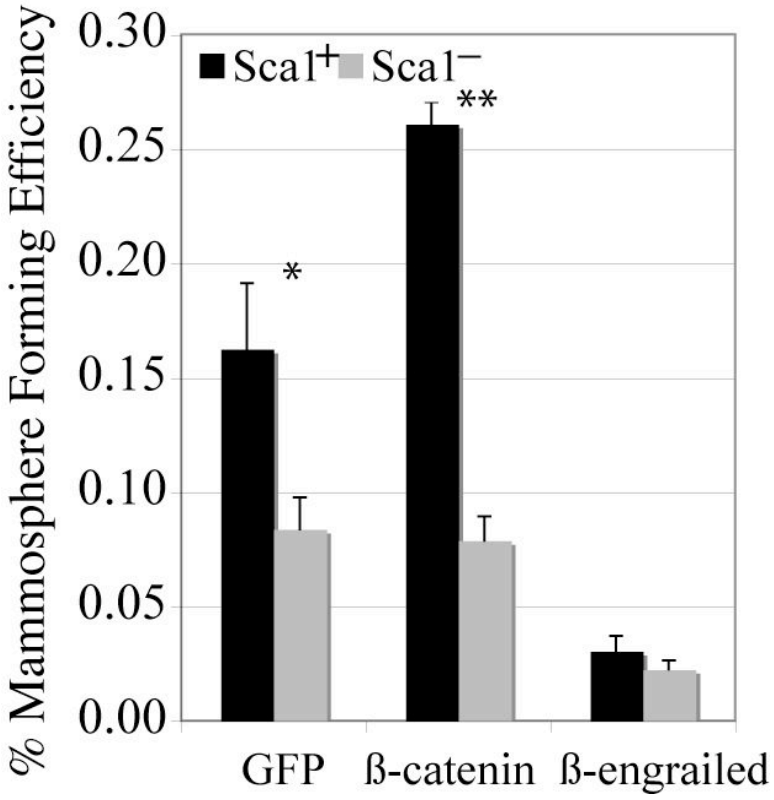

% Mammosphere Forming Efficiency
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Sca1+
Sca1−
*
**
GFP
ß-catenin
ß-engrailed

Figure 4.

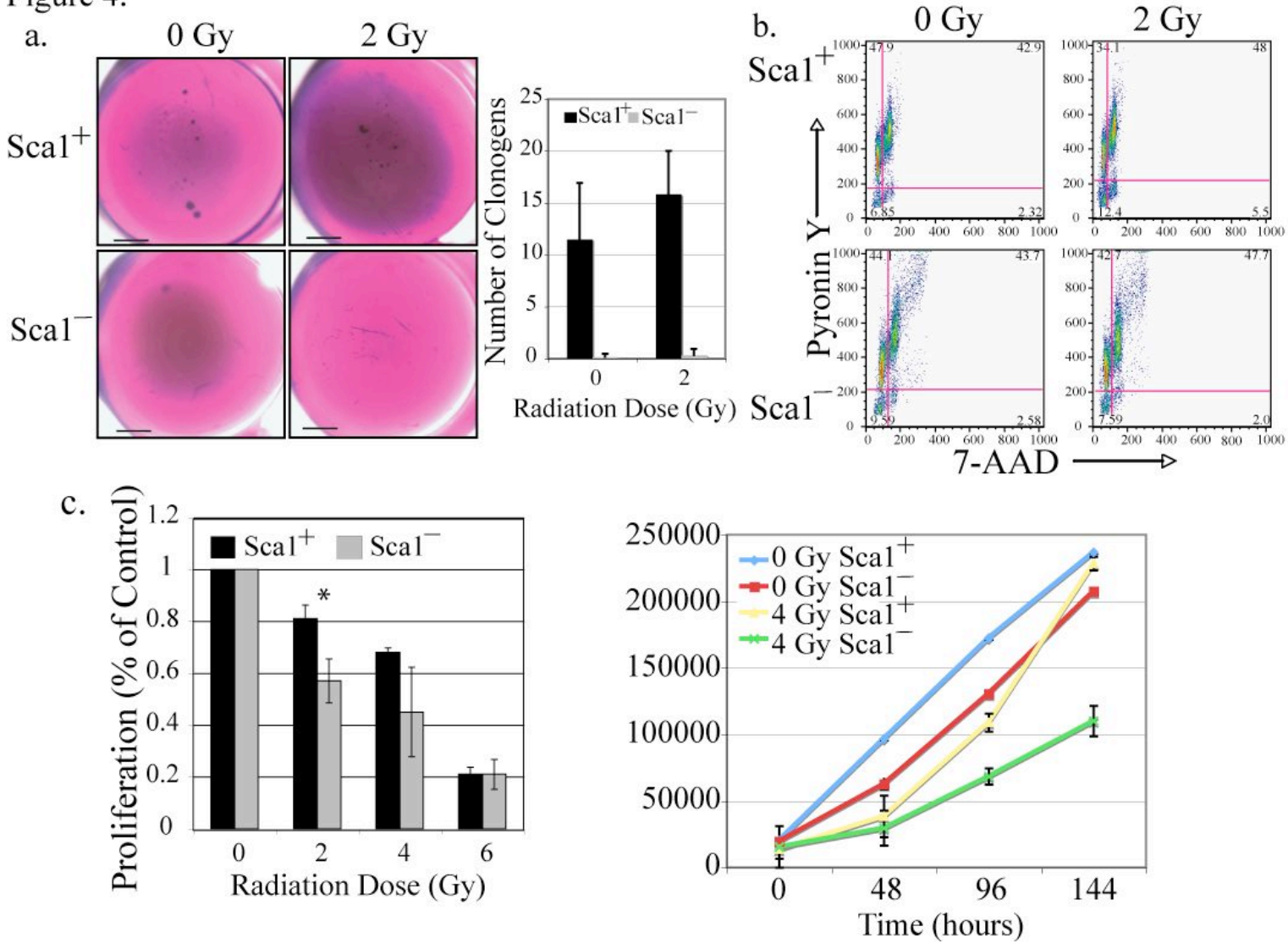

a.
0 Gy
2 Gy
Sca1+
Sca1−
Number of Clonogens
Sca1+ Sca1−
Radiation Dose (Gy)
b.
0 Gy
2 Gy
Sca1+
Sca1−
Pyronin Y
7-AAD
c.
Proliferation (% of Control)
Sca1+
Sca1−
*
Radiation Dose (Gy)
0 Gy Sca1+
0 Gy Sca1−
4 Gy Sca1+
4 Gy Sca1−
Time (hours)

Figure 5.

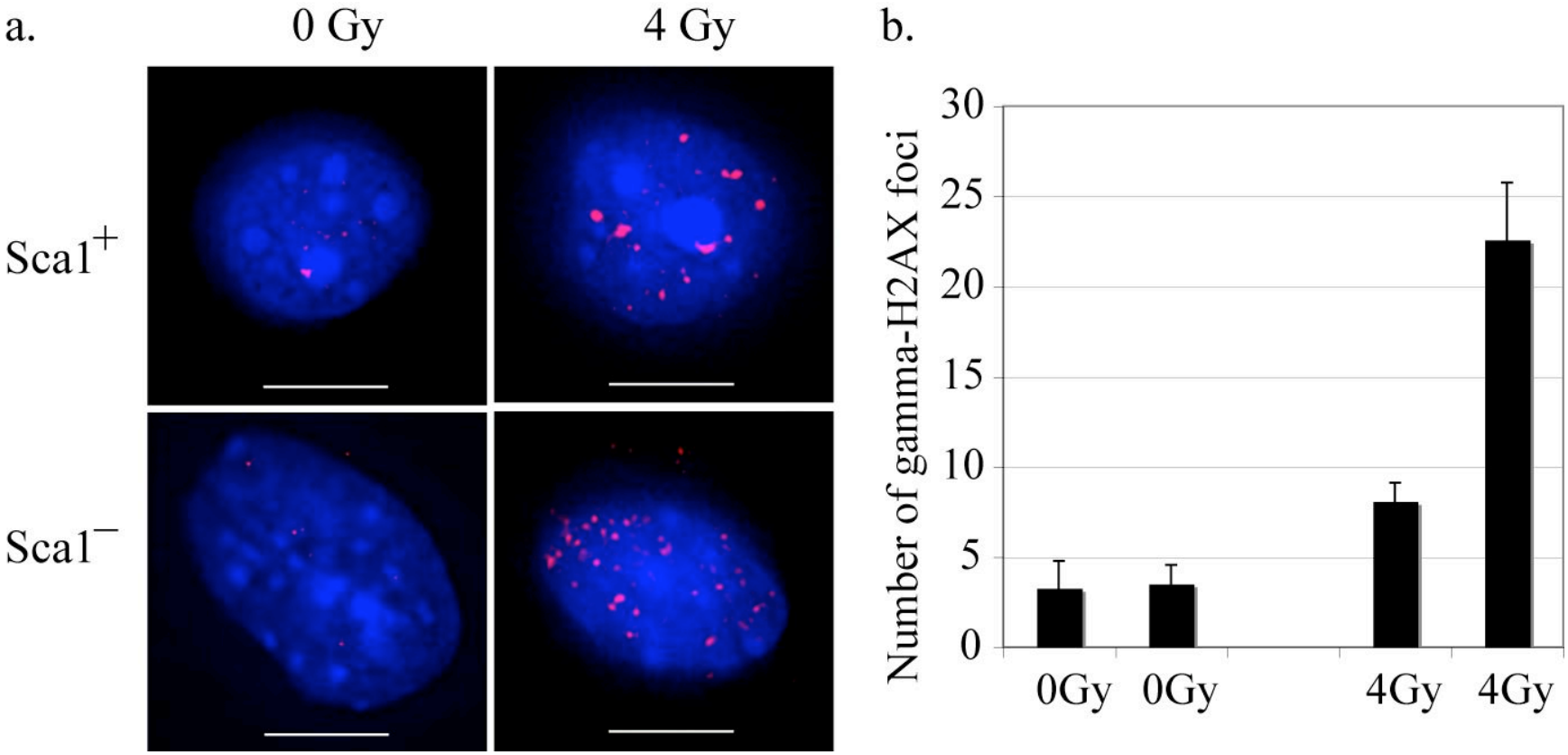
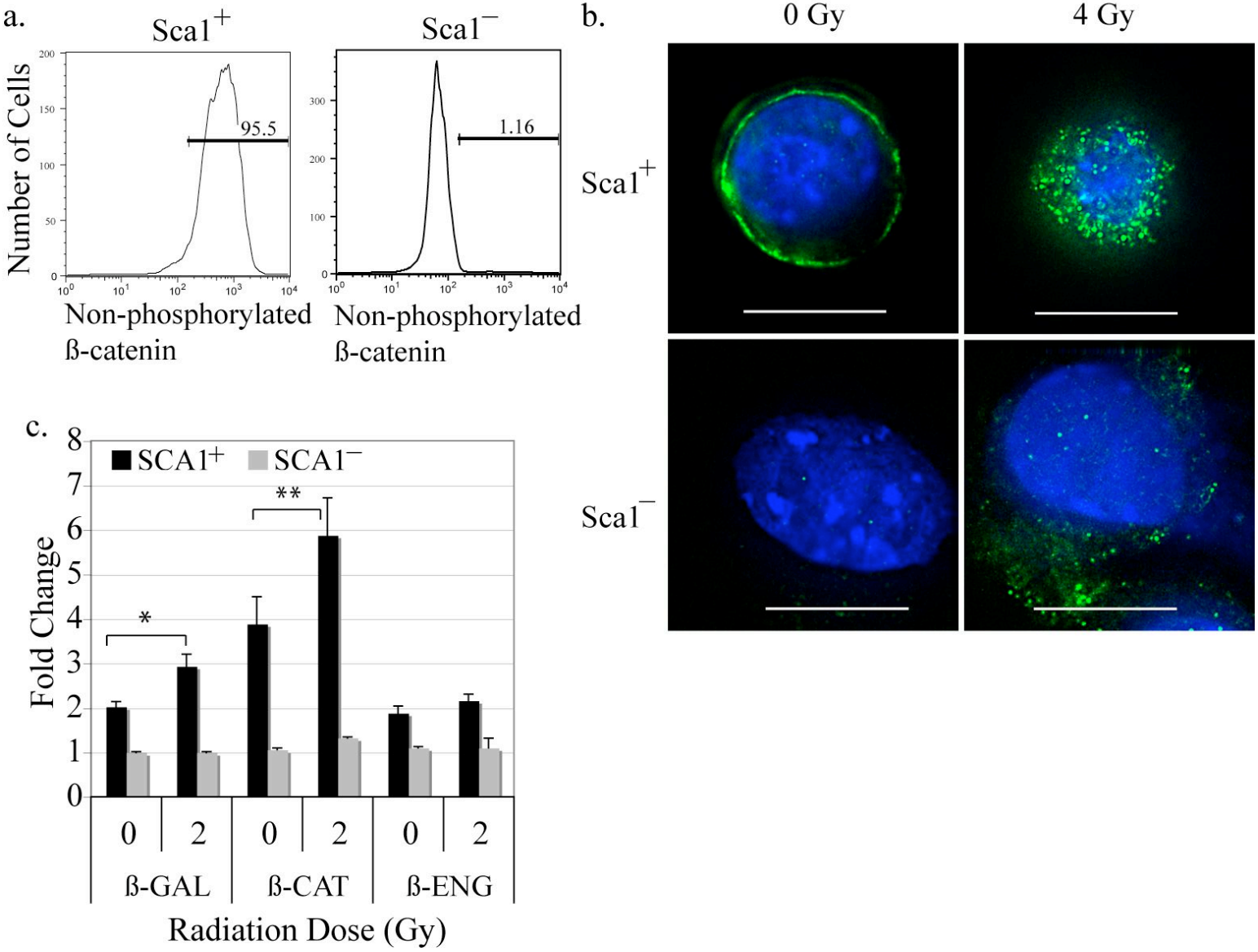

a.
0 Gy
4 Gy
Sca1+
Sca1−
b.
Number of gamma-H2AX foci
30
25
20
15
10
5
0
0Gy
0Gy
4Gy
4Gy

Figure 6.


a.
Sca1+
Sca1−
Number of Cells
95.5
1.16
Non-phosphorylated ß-catenin
Non-phosphorylated ß-catenin
b.
0 Gy
4 Gy
Sca1+
Sca1−
c.
SCA1+
SCA1−
Fold Change
*
**
0
2
0
2
0
2
ß-GAL
ß-CAT
ß-ENG
Radiation Dose (Gy)

On mammary stem cells

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Summary

Mammary gland stem cells are a quiescent and self-renewing population within the mammary gland that are capable of giving rise to the differentiated ductal, alveolar and myoepithelial cells. To identify mammary gland stem cells, several investigators have employed a variety of methods including: non-adherent mammosphere cultures; 5-bromo-2-deoxy-uridine (BrdU) label-retention studies; cell-surface markers, such as Sca1 and CD49f; and Hoechst dye efflux. These methods have helped identify and further characterize signal transduction pathways such as the Notch, Wnt and Hedgehog pathways that may be important for the self-renewal and fate determination of mammary gland stem cells. Stem cells within the mammary gland have been proposed to underpin many types of breast cancer. A better understanding of the signal transduction pathways and the molecules that are responsible for the self-renewal and survival of these cells will be essential in the design of more effective therapies aimed at the eradication of both cancer-initiating cells and breast cancer stem cells.

Key words: SP, Sca1, LRC, Stem cells, α6-integrin, ER

Introduction

Recent discoveries regarding the isolation and characterization of stem cells, the understanding of signaling pathways involved in their self-renewal and survival, and their potential role in diseases such as cancer have turned academic, political and public attention to the rapidly expanding field of stem cell biology. The most primitive stem cells – embryonic stem cells – have extraordinary differentiation potential and can mature into every cell type in a fully developed organism. Adult stem cells make up a small percentage of the cells found in mature organ systems, where they give rise to specific cell types, such as the skin, mammary gland, gut and central nervous system. Adult stem cells are long-lived, generally quiescent cells that generate new stem cells, and thereby maintain the stem cell pool, as well as more committed progeny, which populate the organ through proliferation (Molofsky et al., 2004; Reya et al., 2001). The most primitive adult stem cell population, which is able to give rise to all cell types within the organ, is thought to be maintained by signals found in the local environment – the stem cell niche (Ohlstein et al., 2004; Rizvi and Wong, 2005). When necessary, it can expand to generate a transiently amplified pool of progenitors to re-populate tissues.

Studies of model systems such as the hub cells in the *Drosophila* testis, the terminal filament and cap cells in the fly ovary (Yamashita et al., 2005), the bulge region of the hair follicle (Tumbar et al., 2004) and crypt cells in the gut have begun to provide insights into the stem cell niche (Radtke and Clevers, 2005). Stem cell quiescence in the niche, for example, is thought to be regulated by cell adhesion. This is mediated in part by homotypic interaction of cadherins from the surrounding niche and the stem cells, as well as interactions between integrins on stem cells and the extracellular matrix.

The mammary gland is organized into a tree-like structure composed of hollow branches. These have an inner layer of luminal epithelial cells that face the lumen and are surrounded by an outer layer of myoepithelial cells that secretes the basal lamina separating the mammary parenchyma from the stroma (Richert et al., 2000). Within the mammary arbor, the ductal cells are those that line the ducts of the mammary gland (Fig. 1c,d). Lobular cells form secretory acinar structures at the end of each branch and, upon pregnancy and lactation, become alveolar cells that produce milk proteins. The ability to replenish the mammary gland through cycles of pregnancy, lactation and involution throughout a woman's lifetime is attributed to stem cells that are proposed to reside in the mammary gland (Williams and Daniel, 1983; reviewed by Smith and Chepko, 2001). These cells are proposed to serve three functions: (1) to give rise to the tissues of the adult mammary gland during development; (2) to allow the enormous tissue expansion and remodeling that occurs in the mammary gland during multiple cycles of pregnancy, lactation and involution; and (3) rarely, to serve as a reserve for repair in the event of tissue damage. At the onset of puberty, the immature mammary gland undergoes rapid growth and differentiation at the tip of the terminal end buds (TEBs; Fig. 1b). The cap cell layer surrounding the TEB can take on a myoepithelial lineage or a luminal epithelial lineage, and therefore cap cells are thought to be multipotent stem cells. However, the TEBs are considered to be only a temporary niche since TEBs are transient structures that disappear once the duct reaches the end of the fat pad.

In the late 1950s, DeOme and colleagues elegantly demonstrated the existence of adult stem cells in mammary tissue by limiting-dilution transplantation experiments in which clonal progenitors can generate complete, functional, mammary outgrowths containing ductal, alveolar and

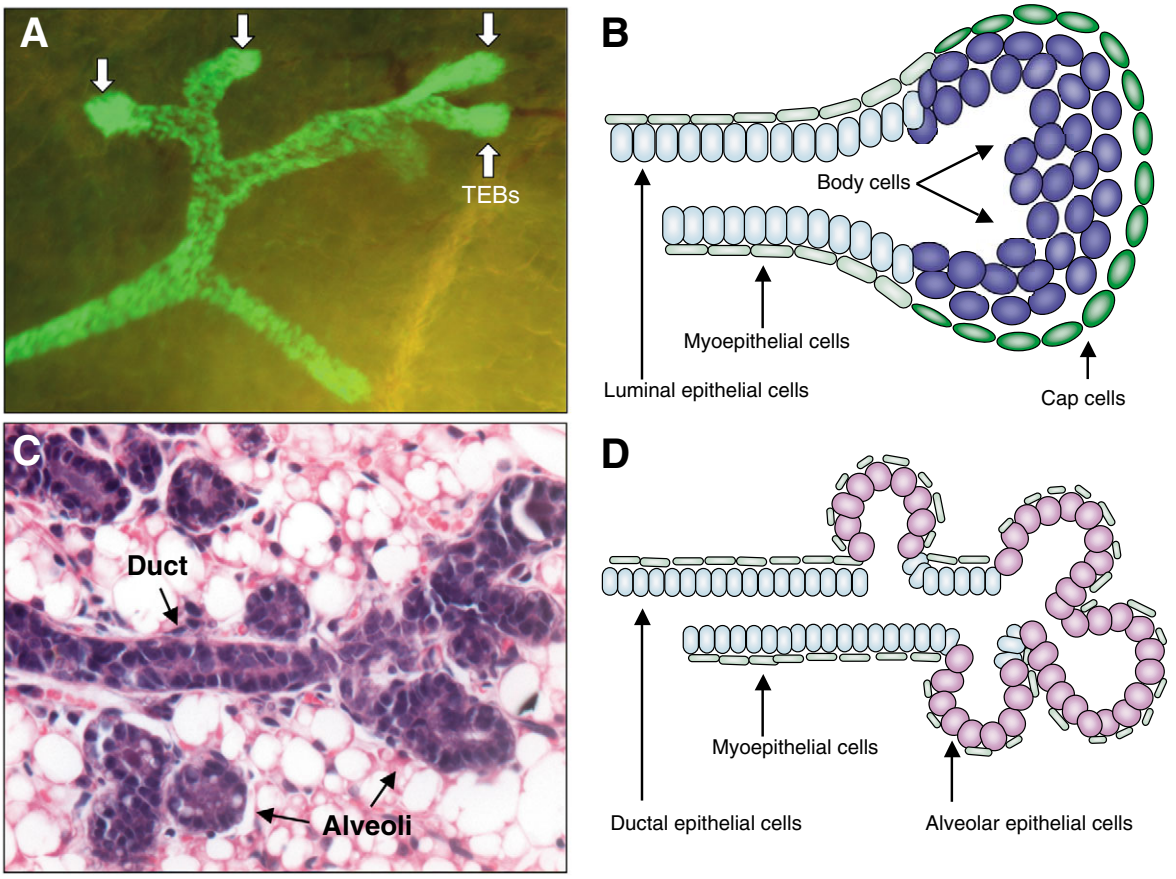

Fig. 1. The terminal end bud (TEB). The TEB appears at the onset of puberty, undergoing rapid growth and differentiation. (A) Expression of Sca1 is enriched in TEBs (arrows) and ducts of six-week-old mice. Micrograph showing live imaging of GFP expression in Sca1-GFP knock-in mice (Sca1-GFP mice kindly provided by T. A. Graubert, Washington University; glands prepared and image captured with help from M. T. Lewis, Baylor College of Medicine). (B) Schematic view of the TEB. A cap cell layer surrounds the body cells. The cap cells can take on either a myoepithelial lineage or a luminal epithelial lineage and therefore are thought to be multipotent stem cells. Differentiated myoepithelial and luminal epithelial cells line the neck of the TEB and the subtending duct. (C) Section, stained with hematoxylin and eosin, of a midpregnant mammary gland from C57BL/6 mice indicating the locations of the ductal and alveolar cells. (D) Schematic view of the ductal and alveolar cells during midpregnancy. The ducts are surrounded by a basal layer of overlapping myoepithelial cells, whereas the alveoli cells are surrounded by a basket-like layer of myoepithelial cells.

myoepithelial cells when transplanted into the cleared mammary fat pads of recipient mice (DeOme et al., 1959). Subsequently, other researchers extended the idea by demonstrating that samples taken from any portion of the mammary gland can give rise to mammary epithelial outgrowths that have complete developmental capacity regardless of their age and developmental stage (Smith and Medina, 1988). This impressive renewal capacity has been ascribed to a multipotent mammary gland stem cell population that resides and persists throughout the mammary parenchyma.

Stem cells are candidates for cells from which cancers originate (Sell, 2004). Bonnet and Dick provided direct evidence for the existence of cancer stem cells in leukemia by showing that only a minority of leukemic cells are pluripotent and can, therefore, reconstitute tumors in the bone marrow of NOD/SCID mice (Bonnet and Dick, 1997). More recently, several groups have prospectively identified cancer stem cells capable of recapitulating solid tumors from which they are derived, including glioma, pediatric meduloblastoma and breast cancer (Al-Hajj et al., 2003; Singh et al., 2003; Singh et al., 2004). Cancer therapy that targets this small population of cancer stem cells might thus be necessary to prevent cancer recurrence (Reya et al., 2001). In this Commentary, we examine current progress in identifying and characterizing adult stem cells in the mammary gland, the pathways responsible for maintaining stem cells in normal mammary tissue and, finally, the role of stem cells and stem cell self-renewal in breast cancer.

Identification of mammary stem/progenitor cell markers*

Several complementary approaches have been employed to isolate, identify and enrich mammary epithelial cells (MECs) that maintain stem/progenitor cell characteristics. Bone fide stem cell markers in general have remained elusive. Until

*Stem cells refer to the most primitive, pluripotent cells. We know that existing surrogate stem cells markers, such as Sca1 or CD49f, are not the only markers that characterize a stem cell. Here, we are designating the cells isolated by the existing surrogate stem cell markers as less primitive progenitor cells instead of stem cells.

recently, there were no known stem/progenitor cell markers in the mammary gland. Therefore, researchers have taken advantage of knowledge obtained in hematopoietic, neural, epidermal and other systems, and applied stem cell markers borrowed from these fields to search for potential stem/progenitor cells in the mammary gland. Below, we focus on recent progress in this area, and readers are referred to a review by Stingl and colleagues for discussion of earlier studies (Stingl et al., 2005).

Sca1

Stem cell antigen 1 (Sca1), a marker of hematopoietic stem cells, is one marker currently used to isolate and enrich for mammary gland progenitors. A population of Sca1$^+$ cells exists in the murine mammary gland (Welm et al., 2002). Label-retention experiments have demonstrated that this population is enriched in slowly dividing, largely quiescent cells (see below). A Sca1-green fluorescent protein (GFP) knock-in approach has shown that Sca1-GFP cells do not co-localize with the progesterone receptor (PR), a marker of differentiation, or peanut lectin, a differentiation marker that interacts with MUC4 on MECs. The highest level of Sca1-GFP expression is in the body cells of the rapidly proliferating TEBs at the tips of the growing ducts (Fig. 1a). In transplantation experiments, Sca1-GFP$^+$ cells isolated by fluorescence-activated cell sorting (FACS), or Sca1$^+$ cells isolated by magnetic bead sorting, have elevated outgrowth activity compared with Sca1-GFP$^-$ cells, which fail to give rise to outgrowths when transplanted into the cleared mammary fat pad. However, like most studies using single markers for FACS analysis, these experiments are highly dependent on the specific cell preparation and gating conditions used to isolate the Sca1$^+$ cells, and there appears to be a gradient of Sca1 expression. Thus, these experiments should not be over-interpreted to indicate that the presence of Sca1 represents an 'all or none' distinction with respect to stem/progenitor cell activity. Indeed, recent experiments have suggested that cells from the COMMA-D mammary epithelial cell line that have high Sca1 expression exhibit increased clonogenicity compared with COMMA-D cells that have intermediate or no Sca1 expression (M. Alfaro and J.M.R., unpublished observations).

Other markers, used to evaluate hematopoietic, epidermal and hepatic stem cells have also been assessed for their ability to allow the differential enrichment of MECs with outgrowth potential, and therefore putative mammary stem cell activity. Stingl and Eaves have reported preliminary evidence, documenting the success of this approach and the likelihood that a multiplicity of markers will be needed to discriminate stem and/or progenitor mammary cells from more differentiated mammary cells (J. Stingl and C. J. Eaves, BC Cancer Research Centre, Vancouver, Canada, personal communication). In addition, their findings suggest that mouse mammary stem cells retain the fluorescent dye Rhodamine-123, which is in contrast to results from adult mouse hematopoietic stem cells that actively efflux this dye. At present, very little is known about the regulation of mammary stem cell proliferation and the potential role regarding their interactions with the niche they occupy in vivo. Identification of surface markers expressed by mammary stem cells is therefore of additional interest because such information might provide clues to the molecular mechanisms involved in their regulation. Furthermore, to date, most of the published transplantation experiments have not looked at the long-term engraftment potential of any of these markers in serial transplantation experiments. Differences in the age and strain of the mice used for these analyses, as well as the methods used to isolate single MECs and the specific antibodies used for FACS analysis, might in part account for the lack of correspondence of Sca1 expression and outgrowth potential between these different studies.

Hoechst dye efflux

The DNA-binding dye Hoechst 33342 has been used as an unique method to identify potential stem cells in a host of tissues, including the bone marrow, heart, lung, muscle, eye and pancreas (Goodell, 2002; Goodell et al., 1996). The dual emission of the Hoechst dye generates a distinct 'side population' from the whole population of cells, which is enriched in Sca$^+$ and lineage$^-$ (B220$^-$, Gr-1$^-$, Mac-1$^-$, CD4$^-$, CD5$^-$ and CD8$^-$) cells. This unique segregation is conferred by the ATP-binding cassette family of multi-drug-resistant transporter proteins, such as the multi-drug-resistant protein (Mdr1 or p-glycoprotein), which actively pump out the Hoechst dye. In fact, when the whole cell population is treated with verapamil, an inhibitor of these transporters, the SP phenotype is lost (Goodell et al., 1996). In the bone marrow, these 'side population' or SP cells, are enriched approximately 1000-fold in hematopoietic stem cell activity in repopulation experiments, and provide an enrichment of 300-fold in radioprotection of lethally irradiated recipients (Goodell et al., 1996; Goodell et al., 1997). In addition, SP cells also contribute to both the myeloid and lymphoid lineages in the transplant recipients.

Rhodamine-123 is another fluorescent dye whose efflux can be used to enrich for potential stem cells. In bone marrow, the percentage of cells in the Rhodamine-123-effluxing subset is similar to that in the Hoechst-dye-effluxing SP cells (i.e. about 10%) (Spangrude and Johnson, 1990). However, the former does not segregate into a population as distinct as the Hoechst-dye-effluxing SP cells and, thus, must be used in combination with other surrogate stem cell markers for stem cell isolation.

Our laboratory has used a similar approach to identify mammary gland Hoechst-dye-effluxing SP (MG-SP) cells (Welm et al., 2002) and has shown that treatment with verapamil blocks their appearance. Interestingly, although the SP phenotype in hematopoietic cells depends on the presence of the ABCG2/BCRP1 (breast cancer resistance protein) transporter (Zhou et al., 2002), deletion of this gene does not lead to loss of the MG-SP population in BCRP1-null mice, which suggests compensation by other ABC transporters, such as Mdr1 (F.B. and J.M.R., unpublished observations). Interestingly, BCRP1 expression has been shown recently to increase in alveolar progenitors and during lactation, perhaps playing a role pumping xenotoxins into milk (Jonker et al., 2005). Mammary gland reconstitution experiments have demonstrated that MG-SP cells retain pluripotent outgrowth potential (Alvi et al., 2003; Welm et al., 2002). However, the Hoechst dye is toxic to MG-SP cells, which has restricted the functional characterization of these cells. Thus, one cannot yet

demonstrate, in limiting-dilution transplantation experiments, enrichment for stem/progenitor cell activity in the MG-SP cells compared with other cells. This MG-SP population is enriched in label-retaining cells (LRCs) compared with the $Sca1^+$ population alone, and is at least fourfold enriched compared with the non-SP population (Welm et al., 2002). LRCs are slowly cycling candidates for stem cells that retain the BrdU label after a prolonged chase. This suggests that the mammary gland contains stem/progenitor cells that have varying degrees of quiescence. However, the LRCs represent only a subpopulation of the MG-SP cells (<10%). Thus, if the MG-SP population represents 1 in 200 of the primary MECs, the MG-SP/LRCs, which may be a more quiescent, primitive population, appear to represent only 1/2000 cells. Interestingly, this is the number of stem cells present in the mammary gland predicted on the basis of limiting-dilution transplantation experiments (Smith and Medina, 1988). Thus, the majority of the MG-SP (and $Sca1^+$ cells) is probably more committed progenitors.

Smalley and colleagues have demonstrated that MG-SP cells exist in human as well as murine MECs (Alvi et al., 2003). By using known epithelial markers, these investigators showed that the MG-SP cells are relatively undifferentiated and express lower levels of cytokeratins K19 and K14 and higher levels of vimentin than non-SP cells. By characterizing in vitro cultures, they found both MG-SP and non-SP cells express K14 and K18, which are markers of myoepithelial and luminal epithelial cells, respectively. Transplantation into cleared fat pads demonstrated that MG-SP cells give rise both to lobuloalveolar and ductal outgrowths, which suggests that the MG-SP cells retain a full differentiative and developmental potential.

Using human cells obtained from mammoplasty reduction, Clayton et al. (Clayton et al., 2004) compared three candidates for stem cell populations: cells co-expressing the luminal and myoepithelial markers EMA and CALLA; EMA^- and $CALLA^-$ cells; and MG-SP cells. The EMA^+ $CALLA^+$ cells do not efflux Hoechst dye, and therefore these are not enriched in the MG-SP. By contrast, within the MG-SP, the majority of cells are EMA^- $CALLA^-$. Furthermore, the majority of the MG-SP cells are $K18^+$ or $K14^+$, and there is an increased proportion of $K18^+$ $K14^+$ cells. The MG-SP might thus be enriched for a population of bipotential cells, able to give rise to both the $K18^+$ luminal and the $K14^+$ myoepithelial lineages. This study suggested that these three populations represent three distinct cell lineages. The EMA^+ $CALLA^+$ population represents the more committed cell fate, ultimately becoming either luminal or myoepithelial cells. Whereas the EMA^- $CALLA^-$ population, lacking specific epithelial markers, represents a more primitive progenitor, capable of giving rise to both luminal and myoepithelial cell types.

Label-retention studies

Several investigators have used label-retention studies to identify mammary stem cells (Smith, 2005; Welm et al., 2002; Zeps et al., 1998). By labeling 4-week-old virgin mice with BrdU for 2 weeks (a time at which the TEBs are maximally active) and chasing the label for 9 weeks (during which ductal morphogenesis is completed), our laboratory was able to identify a small fraction of BrdU-LRCs in the total population of epithelial cells. Very few of these LRCs express differentiation markers, such as PR, which suggests that they represent a less differentiated state. The LRCs are twice as enriched in the MG-SPs compared with the $Sca1^+$ population, which supports the idea that the mammary gland contains stem/progenitor cells that have varying degrees of commitment. In general, the MG-SPs appear to represent a population of more-primitive stem/progenitor cells, whereas the majority of the $Sca1^+$ population might represent more-committed progenitors. However, since approximately 75% of the MG-SP cells are $Sca1^+$, a subset of these cells clearly overlap (Welm et al., 2002), and both the MG-SP and the $Sca1^+$ population appear to represent cells that have a range of activities.

More recently, Smith (Smith, 2005) asked whether mammary LRCs retain their template DNA strand and pass their newly synthesized chromatids to their daughter cells during asymmetric divisions, an idea originally proposed by Cairns (Cairns, 1975) and later by Potten et al. (Potten et al., 1978; Potten et al., 2002). Smith labeled mice receiving transplanted mammary tissue with [^{3}H]-thymidine (^{3}HTdR) for 5 days, chased for 3-4 weeks and, towards the end of the chase, gave a pulse of a second label, BrdU. LRCs retaining the ^{3}HTdR label (template strand) again were detectable in the mammary gland, and a large percentage of the ^{3}HTdR cells incorporated BrdU. Following the chase, the level of the BrdU label decreased in the daughter cells whereas the ^{3}HTdR was retained. This result suggests that the mammary LRCs selectively retain their ^{3}HTdR-containing template strand, while passing on the newly synthesized BrdU-labeled daughter strand to their progeny during asymmetric divisions. Furthermore, by transplanting LacZ-marked epithelial cells in a similar experiment, Smith demonstrated that the mammary gland stem cells can undergo self-renewal as well as asymmetric division in mammary gland outgrowths. The identification of a population of actively dividing LRCs demonstrated by the incorporation of BrdU indicates that these cells are not totally quiescent.

Hormone receptor status

One unique aspect of mammary gland development is its dependence on the circulating steroid hormones estrogen and progesterone. Furthermore, the majority of breast cancers are estrogen receptor (ER) positive and are responsive to hormonal therapy (Allred et al., 2004; Sorlie et al., 2003). Thus, whether mammary epithelial stem cells express steroid receptors such as ERα or PR is, therefore, a critically important question relevant to the etiology of ER-positive and ER-negative breast cancers. Clarke and colleagues (Clarke et al., 2005) have used several complementary approaches to characterize human breast epithelial stem cells with respect to ERα and PR. First, using long-term [^{3}H]-labeling of human breast epithelial xenografts implanted in athymic nude mice, they showed that the LRCs co-express putative stem cell markers such as $p21^{CIP/WAF1}$ and Msi1, an ortholog of the *Drosophila* Musashi protein involved in asymmetric stem cell division. A proportion of the cells also express steroid receptors. Next, by co-staining cells in the mammary gland for K19, a putative stem cell marker, and steroid receptors, they observed that $K19^+$ cells frequently express steroid receptors and, conversely, steroid-receptor-positive cells in the gland are likewise $K19^+$. Finally,

by analyzing Hoechst dye efflux, the authors observed that steroid-receptor-positive cells are highly enriched in the MG-SP cells compared with the non-SP population. Furthermore, the steroid-receptor-positive MG-SP cells can generate branching structures that include myoepithelial as well as luminal epithelial cell types, when grown on matrigel. Accordingly, Clarke et al. (Clarke et al., 2005) suggested that steroid-receptor-positive cells are enriched in breast epithelial stem cells with the capacities for self-renewal and differentiation.

Mammospheres

Neural stem cells cultured in suspension form clusters of apparently homogenous cells called 'neurospheres', which display an increased capacity for self-renewal. Using an analogous approach, Wicha and colleagues have developed a method to enrich for mammary stem cells in an undifferentiated state by culturing cells under anchorage-independent conditions (Dontu et al., 2003). The 'mammospheres' contain $CD49f^+$, $K5^+$ and $CD10^+$ cells. A few cells express luminal and myoepithelial markers, such as ESA and K14. When grown on a collagen substratum, mammosphere-derived single cells differentiate into colonies that express markers specific for only ductal or myoepithelial cells, or markers of both cell lineages. When grown on matrigel, the single cells can differentiate into functional complex branching structures similar to ductal and alveolar structures. In addition, when treated with prolactin, these mammospheres form functional alveolar cells that secrete β-casein into the lumen. By growing mammosphere-derived single cells, not only did these investigators show bipotential (giving rise to both luminal and myoepithelial cell types) and tripotential (giving rise to luminal, myoepithelial and alveolar cell types) differentiating capacity, they also demonstrated by retroviral tagging that mammospheres are clonally derived. In addition, they demonstrated that these cells can be propagated through multiple passages in an undifferentiated state and retain their multipotent capacity.

Cancer stem cells

Mutations that initiate breast cancer appear to accumulate slowly in cells that persist throughout a woman's lifetime, since there is an exponential increase in breast cancer incidence with age, and since girls exposed to excess radiation in adolescence have an increased risk of breast cancer 20-30 years after the exposure. It has been hypothesized that delayed cancers result from damage to a quiescent cell with unlimited potential for self-renewal that may persist for decades and ultimately give rise to a malignancy in response to an unknown proliferative signal. For this reason, stem cells make an attractive candidate for the cellular origin of cancer since they possess many features of the tumor phenotype, including self-renewal and essentially unlimited replicative potential (Reya et al., 2001).

Until recently, the prospective identification of tumor stem cells, which are a limited population of tumor cells responsible for giving rise to all components of a heterogeneous tumor, had remained elusive. However, Clarke and colleagues (Al-Hajj et al., 2003) have now used cell-surface markers to isolate a subpopulation of highly tumorigenic breast cancer cells from the bulk of human breast tumor cells. They observed that $CD44^+$ $CD24^-$ human breast tumor cells have an increased ability to form tumors when injected into the cleared mammary fat pad of etoposide-treated NOD/SCID mice, and that although as few as 100 $CD44^+$ $CD24^-$ human breast tumor cells can re-capitulate the human tumors from which they are derived, injection of 10,000 cells of other phenotypes fails to give rise to tumors. Tumors arising from $CD44^+$ $CD24^-$ cells are heterogeneous, giving rise to tumorigenic cells and a population of non-tumorigenic cells. In addition, the $CD44^+CD24^-$ cells can propagate indefinitely. Therefore, this subpopulation possesses stem cell characteristics, such as the ability to self-renew and to give rise to multipotent progenitors. Although these authors were unable to demonstrate tumor outgrowth from a single tumor stem cell, these data significantly advanced the hypothesis that tumor stem cells exist in human solid tumors and underscore the importance of better understanding of stem cell biology in the treatment of human tumors.

Signaling pathways implicated in stem cell self-renewal

Understanding the signaling pathways involved in the self-renewal of both normal and cancer stem cells is an important first step towards anti-cancer therapies targeting cancer stem cells. Studies of hematopoietic, intestinal, muscle and embryonic stem cell models have identified several key signaling pathways involved in self-renewal and maintenance of the stem cell pool (Yamashita et al., 2005). These include the Wnt/β-catenin, Notch, Hedgehog (Hh), transforming growth factor (TGF)-β, PTEN and Bmi signaling pathways (Andl et al., 2002; Boulanger et al., 2005; Brennan and Brown, 2004; Dontu et al., 2004; Hatsell et al., 2003; Ingham and McMahon, 2001; Korinek et al., 1998; Leung et al., 2004; Lewis et al., 1999; Machold et al., 2003; Molofsky et al., 2004; Reya et al., 2003; Stiles et al., 2004). Unsurprisingly, many of these pathways have been implicated in cancer, which is consistent with the hypothesis that dysregulation of normal stem cell self-renewal can lead to cancer initiation.

In the mammary gland, increasing evidence supports a role for Wnt/β-catenin, Notch and Hh signaling pathways in mammary stem/progenitor cell self-renewal. In addition, Deugnier et al. (Deugnier et al., 2002) have suggested that epidermal growth factor (EGF) signaling controls the developmental potential of transplanted murine mammary BC44 cells (a clonal derivative of HC11 mouse mammary epithelial cells, which express basal markers). This is interesting given the impressive pre-clinical and clinical data suggesting EGF receptor inhibitors potentiate the effects of radiation and improve overall survival in some cancers (Harari, 2004).

Wnt/β-catenin

Wnt is a secreted protein that binds to its receptor – frizzled (FZD) – and leads to the stabilization and translocation of β-catenin into the nucleus, where it binds the LEF/TCF transcription factors (Moon et al., 2002). Wnt signaling is involved in patterning during development and components of the pathway are mutated in several cancers, including

colorectal cancer, desmoid tumor and hepatoblastoma (Beachy et al., 2004). The *Wnt* gene was originally identified as a viral insertion in mouse mammary tumor virus- (MMTV) (Nusse and Varmus, 1992) induced mammary tumor. Stabilization of β-catenin has been demonstrated in >50% of human breast cancers, although overt mutations of pathway components in breast cancer have yet to be identified (Brennan and Brown, 2004). Loss of Wnt inhibitors such as SFRP1 and increased levels of β-catenin are associated with poor prognosis in breast cancer (Klopocki et al., 2004; Ugolini et al., 2001). In addition, Jain and colleagues have demonstrated that even transient loss of expression of the Wnt downstream target gene, *Myc*, can lead to irreversible loss of malignant cells (Jain et al., 2002). Recent studies also suggest that autocrine Wnt signaling plays a role in several human cancer cell lines, including breast and ovarian lines (Bafico et al., 2004).

β-catenin has been implicated as a stem cell survival factor in several systems, including neural crest cells, gastrointestinal crypts, epidermal follicles and hematopoetic stem cells (Reya et al., 2003). Inhibition of β-catenin signaling in mammary alveolar progenitors blocks mammary development and pregnancy-induced proliferation, implicating β-catenin as a stem cell survival factor in the mammary gland (Tepera et al., 2003). Alexander and colleagues provided the first direct evidence of Wnt signaling pathways in the maintenance of the stem/progenitor pool in the non-neoplastic mammary gland (Liu et al., 2004). They showed that the SP-enriched progenitor fraction is increased in the mammary gland of MMTV–Wnt-1 and MMTV–ΔNβ-catenin transgenic mice, and that ectopic Wnt-3a increases the SP fraction in MECs after 3 days in culture. The SP fraction in MECs expands in culture in response to radiation treatment, and this effect is significantly increased in MECs from MMTV–Wnt-1 mice. This suggests that Wnt signaling can mediate radiation resistance of the progenitor fraction in non-neoplastic MECs (W.A.W., M.S.C., F.B., J.M.R. and M. P. Alfaro, unpublished observations).

Recent studies have also demonstrated a role for Wnt signaling in neoplastic mammary stem-cell-like progenitors (Li et al., 2003). Li et al. have demonstrated an expansion of $Sca1^+$ progenitor cells in pre-neoplastic and neoplastic mammary gland lesions from MMTV–Wnt-1 mice and other transgenic mice in which the Wnt pathway is active, but did not observe this in other mammary tumor models in which the Ras pathway is activated (Li et al., 2003). In addition, these studies suggested that $K6^+Sca1^+$ cells present in neoplastic mammary lesions from Wnt-1-transgenic mice might represent bipotent cells, in this case capable of giving rise to both luminal and myoepithielial tumor cells, which represent target cells for stochastic mutations that result in mammary tumorigenesis.

Although the phosphatase PTEN, a tumor suppressor mutated in almost as many cancers as p53 (Stiles et al., 2004), has been implicated in stem cell renewal in embryonic stem cells, few studies have focused on its role in stem cell renewal in the mammary gland. Li et al. (Li et al., 2003) have demonstrated loss of PTEN heterozygosity in tumors derived from the progeny of MMTV–Wnt-1 mice crossed with $PTEN^{+/-}$ mice. Interestingly, the loss of PTEN occurs in both the luminal and myoepithelial lineages, which suggests that this occurs in a bipotent progenitor, perhaps analogous to the $K14^+K18^+$ cells observed in the SP population by Vivanca and colleagues (Clayton et al., 2004). The PTEN signaling pathway interacts with numerous signaling pathways important for development and can affect Wnt signaling indirectly through stabilization of the β-catenin cytosolic pool (Stiles et al., 2004). Therefore, the role of the interaction between Wnt and PTEN signaling in mammary stem/progenitor cell renewal remains to be elucidated.

In addition to this recent evidence for Wnt/β-catenin signaling in normal and neoplastic adult mammary stem/progenitor cells, there is substantial evidence for Wnt/β-catenin signaling in the developing mammary gland (Howe and Brown, 2004). Knockout mice lacking the Wnt/β-catenin pathway transcription factor LEF (van Genderen et al., 1994) and mice expressing the Wnt signal inhibitor Dickkopf driven by the K14 promoter fail to develop mammary glands (Andl et al., 2002). These data support the speculation that Wnt is necessary for maintenance of the stem cell pool in the mammary bud (Brennan and Brown, 2004; Korinek et al., 1998).

Notch

The interaction of Notch receptors with their ligands (Delta-like-1, -3 or -4 and/or Jagged-1 or -2) promotes cleavage of the intracellular domain. This involves the ADAM protease family and γ-secretase, and allows the intracellular domain of Notch to translocate to the nucleus and act on downstream target genes (Weng, 2004). Notch 4 is important both in normal mammary gland development (Smith et al., 1995) and was identified as an MMTV insertion site in mammary tumors in mice (Gallahan and Callahan, 1997). Accordingly, transgenic mice carrying constitutively active Notch 4 develop mammary tumors (Callahan and Egan, 2004). Dontu and colleagues have examined the role of Notch in the formation of mammospheres from human MECs derived from mammoplasty specimens (Dontu et al., 2004). A synthetic notch ligand shown to induce luciferase activity from the Hes promoter, a known downstream target of Notch signaling, increases secondary mammosphere formation tenfold. Conversely a Notch-4-blocking antibody completely abrogates secondary mammosphere formation (Dontu et al., 2004). These data from human specimens are the first to demonstrate directly a role for Notch signaling in stem cell renewal in the mammary gland.

Hedgehog

Regulation of Hh signaling occurs during normal development of virtually every organ system, including the mammary gland (Bailey et al., 2000; Cohen, 2003), and components of this pathway have been shown to be mutated or overexpressed in multiple cancers, including breast cancer, basal cell carcinoma, medulloblastoma, fibrosarcoma and rhabdosarcoma (Beachy et al., 2004). The core components of the Hh signaling network (Lewis et al., 2001) include ligands (Sonic hedgehog, Shh; Indian hedgehog, Ihh; and Desert hedgehog, Dhh), receptors (Patched-1 and -2, Ptc1 and 2), effector (Smoothened, Smo) and transcription factors (e.g. Gli1-3). In the central nervous system, Hh is required for neural stem cell proliferation in neurospheres, and inactivation of smoothened inhibits proliferation of neural stem cells in vivo and in vitro (Machold et al., 2003). Hh is also required for proliferation of somatic ovarian stem cells in *Drosophila* (Zhang and Kalderon, 2001).

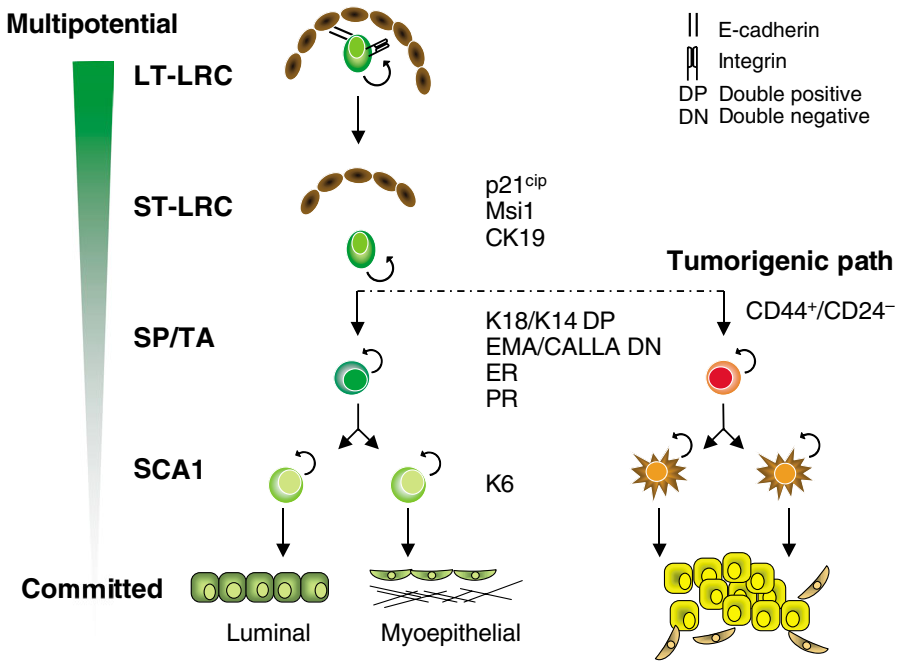

Fig. 2. Mammary gland stem/progenitor-cell fate. The degree of stemness potentially decreases from top to bottom: as the cell becomes more committed, the cell gradually loses its stemness. The stem cells are able to self-renew and proliferate within the niche, maintained in their un-differentiated state by cell-matrix and cell-cell interactions with the niche cells, involving integrins and cadherins, respectively. These cells can be distinguished by their long-term label-retaining cell (LT-LRC) properties, which are thought to reflect a state of quiescence. Responding to stimuli, stem cells exit the niche by becoming short-term (ST)-LRCs. These actively cycle and express stem cell markers such as $p21^{cip}$, Msi1 and CK19. As they become further committed, they become the transit-amplifying progenitors (TAs), comprising the side population (SP) that are able to efflux the Hoechst dye. The SP/TAs express bipotential markers, such as $K18^+$ and $K14^+$, or EMA^- $CALLA^-$, and may be steroid receptor positive. The SP/TA cells eventually give rise to more committed progenitors that are $Sca1^+$. The $Sca1^+$ population differentiates into luminal and myoepithelial cells. Stem cells are thought to possess many of the features that constitute the tumor phenotype, including self-renewal and unlimited replicative potential. Tumorigenic mutations are presumably sustained in the expanding SP/TA population. These cells give rise to tumorigenic progenitor cells. $CD44^+$ $CD24^-$ may be markers that distinguish tumorigenic progenitor cells from normal progenitor cells.

Studies of mammary gland ductal morphogenesis provide support for a role for Hh in interactions between the stroma and epithelial cells in the developing mammary gland (Gallego et al., 2002; Lewis et al., 1999; Lewis et al., 2001). In addition, recent studies have suggested that Hh signaling is activated in a majority of human breast cancers, based on immunohistochemical staining showing uniform overexpression of PTC1 and nuclear GLI1 (both markers for activated Hh signaling) in a set of 52 invasive breast cancers (Kubo et al., 2004). Furthermore, the Hh inhibitor cyclopamine can inhibit growth of a subset of breast cancer cell lines in vitro (Kubo et al., 2004). Preliminary studies in several laboratories have also suggested that the Hh pathway also plays a critical role in mammary stem cell self-renewal (G. Dontu and M. Wicha, personal communication; M. T. Lewis and J.M.R., unpublished observations).

A model for stem cell progression

The work of several laboratories has identified several distinct populations of stem/progenitor cells that display different degrees of commitment (Fig. 2). The [^{3}H]TdR-LRCs that do not retain BrdU, which we can term long-term LRCs (LT-LRCs), might represent the most primitive, quiescent, template-retaining stem cells present in the stem cell niche. Short-term LRCs (ST-LRCs) that actively cycle and are labeled by BrdU, but retain their original DNA template strand, would represent the next level. The heterogeneous MG-SP population appears to represent primarily a transient-amplifying population (SP/TA), but is also enriched in LRCs. It is more enriched in LRCs than the $Sca1^+$ population and, therefore, might be less differentiated than both the $Sca1^+$ and the EMA^+ $CALLA^+$ populations. To determine where cancer stem cells fit into this lineage, we have extrapolated from studies in the hematopoietic field to suggest that mammary gland stem cells are sequestered in a stem cell niche where their quiescence is maintained by adhesion. Increased activation of certain oncogene products, including Myc (Wilson et al., 2004), possibly as a result of the activation of the canonical Wnt/β-catenin signal transduction pathway, may decrease adhesion in asymmetrically dividing daughter cells. Once the stem cells exit the niche, they might become actively dividing early progenitor cells that retain their parental DNA template strand; subsequently, the more committed progenitors, TA cells, no longer retain the template DNA strand and continue to expand. These cells may then accumulate oncogenic mutations and be the primary targets for tumorigenesis.

Clinical implications

Data identifying cancer stem cells in leukemia and solid human tumors such as medulloblastoma, glioma and breast cancer highlight the need for a dramatic shift in the way we design cancer therapies. Since a small population of cancer stem cells can recapitulate the entire tumor, we must assess the efficacy of current cancer therapies at eradicating this small population, which probably drives cancer recurrence.

Clinically, radiation therapy is typically given in small daily doses to reduce normal tissue toxicity yet still achieving adequate tumor cell kill. Radiobiology studies from the 1980s demonstrated that tumors can undergo accelerated repopulation between daily fractions of radiation dose in both

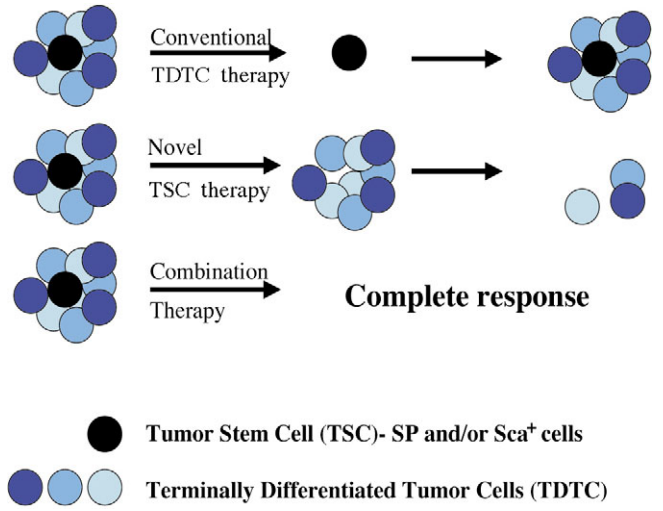

Fig. 3. Cancer therapy that does not kill tumor stem cells may provide gratifying initial results but ultimately result in recurrence. Conventional therapies target proliferating, terminally differentiated cells may leave tumor stem cells, which could lead to recurrence. Ideally, tumor stem cell therapies would specifically target tumor stem cells. Used alone, they might lead to tumor regression, but not dissolve tumor bulk, leading to questions regarding response rates and potentially untreated tumor-related symptoms. Combining conventional therapy with treatment targeting tumor stem cells may effectively eliminate both tumor bulk and tumor stem cells that might otherwise lead to recurrence.

in vivo and in vitro tumor models (Thames et al., 1996). This effect was demonstrated in clonogenic assays in which small single doses of radiation increased the number of tumor clonogens. An understanding of this biological phenomenon led to randomized trials of altered fractionation radiation therapy schedules, such as concomitant boost, whereby the last week of radiation therapy includes a second daily fraction during the fifth week to counteract the effect of accelerated repopulation. This scheme has been shown in a multi-center Phase III randomized clinical trial to improve overall survival in head and neck cancer (Fu et al., 2000). These data support the hypothesis that the clinical effect of accelerated repopulation derives from tumor stem cell clonogens responding to cellular stress that results from either radiation or potentially chemotherapy. Tumor stem cells might be more resistant to radiation than the differentiated cells that make up the bulk of the tumor, and it is possible that it is the resistant tumor stem cells remaining after definitive therapy that ultimately self-renew and amplify to give rise to tumor recurrence.

This phenomenon might apply in the mammary gland. Treatment of Sca1$^+$ immortalized mouse mammary cells with either taxol or radiation leads to an increase in the number of clonogens formed in matrigel, which is consistent with the hypothesis that progenitor cells are resistant to radiation and might expand in response to radiation (W.A.W. et al, unpublished observations). Interestingly, Ly-6E.1, a human ortholog of Sca1, is a marker of advanced tumorigenicity and upregulated in response to stress such as heat shock or serum starvation (Treister et al., 1998). Conventional cancer therapy that targets proliferating, terminally differentiated cells with limited replicative potential may initially lead to a favorable clinical response but fail to eliminate the small population of cancer stem cells that underpin recurrence. Thus, investigation of the mechanisms and signaling pathways that support stem cell renewal in normal and malignant tissue may provide new targets for therapies designed to complement existing approaches and reduce tumor recurrence (Fig. 3).

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